Exhibit A

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Immunology

Functional immunoglobulin M production after transfection of cloned immunoglobulin heavy and light chain genes into lymphoid cells

(protoplast fusion/G418 selection)

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ABSTRACT The rearranged immunoglobulin heavy (μ) and light (κ) chain genes cloned from the Sp6 hybridoma cell line producing immunoglobulin M specific for the hapten 2,4,6-trinitrophenyl were inserted into the transfer vector pSV2-neo and introduced into various plasmacytoma and hybridoma cell lines. The transfer of the μ and κ genes resulted in the production of pentameric, hapten-specific, functional IgM.

Work over the last decades has provided extensive information on immunoglobulin function and structure (1). Despite this information, it has been possible only in gross terms to relate molecular function with particular structural features.

With the advent of genetic engineering and gene transfer techniques, questions regarding structure–function relationships can now be readily addressed—that is, virtually any gene segment can be modified precisely *in vitro* and the novel segment can then be exchanged with its normal counterpart. By introducing such engineered genes into the appropriate cells, the effects of systematic alterations in protein structure on protein function can be assessed.

Because immunoglobulin production is a specialized function of cells of the B-lymphocyte lineage, it is expected that the conditions for proper Ig gene expression will be provided only in appropriate immunocompetent cells. For example, to produce normal pentameric IgM(κ), a cell must transcribe, process, and translate RNA for the μ and κ chains and also provide J protein, enzymes for the proper polymerization and glycosylation of the Ig chains, as well as a suitable secretory apparatus. We have previously described a system for transferring a functional immunoglobulin κ light chain gene into IgM-producing hybridoma cells (2). Here we extend this work to show that the transfer of the μ and κ chain genes of a defined specificity into various plasmacytoma and hybridoma cell lines results in the production of functional pentameric, hapten-specific IgM(κ).

MATERIALS AND METHODS

Cell Lines. X63Ag8 was originally derived (3) from the plasmacytoma MOPC21 and synthesizes IgG1(κ) of unknown specificity. X63Ag8.653 was derived from X63Ag8 as a subclone that synthesizes neither the heavy (γ1) nor light (κ) chain (4). Similarly, Sp2/0Ag14 is an Ig nonproducing subclone of the Sp2 hybridoma (5). Sp6 is a hybridoma making IgM(κ) specific for the hapten 2,4,6-trinitrophenyl (TNP); originally this cell line produced the γ1 and κ chains of X63Ag8 as well as the (TNP specific) μ_{TNP} and κ_{TNP} chains (6). A subclone of Sp6 not making the γ1 chain was isolated, and the Sp602 and Sp603 cell lines were derived from this γ1 nonproducer. The mutant cell line Igm-10, derived from Sp602 (7), lacks the gene encoding μ_{TNP} (8).

Gene Transfer. The construction of pSV2-neo plasmid vectors carrying the genes for μ_{TNP} or κ_{TNP} or both is described in the text. The vectors were transfected into the $r_k^-m_k^-$ *Escherichia coli* strain K803. To transfer the vector, bacteria bearing the appropriate plasmids were converted to protoplasts and fused to the indicated cell lines as described (2). The frequency of G418-resistant transformants per input cell was approximately 10^{-4} for X63Ag8 and Sp2/0Ag14, 10^{-5} for Igm-10, and 10^{-4} for X63Ag8.653.

Analysis of Ig. As described previously (7), Ig was biosynthetically labeled, in the presence or absence of tunicamycin, immunoprecipitated, and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis with or without disulfide bond reduction. TNP binding IgM was assayed by TNP-dependent hemagglutination and by TNP-dependent enzyme-linked immunoadsorbent assay (ELISA) as described (2, 7). The hemolyses of protein A-coupled erythrocytes and TNP-coupled erythrocytes were used to assay total IgM- and TNP-specific complement activating IgM, respectively (7).

Analysis of RNA and DNA. Cytoplasmic RNA was isolated according to Schibler *et al.* (9) and subjected to RNA blot analysis as described by Thomas (10).

Procedures for DNA extraction (11), nitrocellulose blotting (12), and radiolabeling of probes (13) have been described (14, 15). Probes specific for genes encoding immunoglobulin constant and variable regions are detailed in the figure legends.

RESULTS

Description of Vectors and Expression Systems. The hybridoma cell line Sp6 secretes IgM(κ) specific for the hapten TNP. We have previously described the cloning of the TNP-specific κ gene, designated Tκ1 (16), and the construction of the recombinant, pR-Tκ1, where Tκ1 is inserted in the *Bam*HI site of the vector pSV2-neo (2, 17). The μ_{TNP} gene was cloned in ACh4A from *Eco*RI partially digested DNA of Sp6 cells, and this clone is designated Sp6-7.18. The 16-kilobase-pair (kbp) fragment carrying the variable and constant regions was obtained from Sp6-7.18 after partial digestion with *Eco*RI and was inserted at the *Eco*RI site of the vectors pSV2-neo and pR-Tκ1. In these recombinants, designated pR-Sp6 and pR-HL$_{TNP}$, re-

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Abbreviations: TNP, 2,4,6-trinitrophenyl; ELISA, enzyme-linked immunoadsorbent assay; kbp, kilobase pair(s); SV40, simian virus 40; kb, kilobase(s).

spectively, the μ_{TNP} gene lies in the same orientation as the κ_{TNP} gene in pR-TκI—i.e., the direction of transcription of μ_{TNP} is opposite that of the simian virus 40 (SV40) early promoter (Fig. 1).

The mutant cell lines igk-14 and igm-10 that lack the κ_{TNP} gene and μ_{TNP} gene, respectively, were originally isolated from sublines of Sp6 (7). We have previously used igk-14 as a recipient cell line to assay expression of the κ_{TNP} gene (3). Expression of the μ_{TNP} gene of pR-Sp6 was assayed here in igm-10. The simultaneous production of both μ_{TNP} and κ_{TNP} chains from the vector pR-HL_{TNP} is assayed in X63Ag8, the IgG1-producing plasmacytoma parent of the Sp6 hybridoma. In later experiments the pR-HL_{TNP} vector was assayed in the non-producing cell lines Sp2/0Ag14 and X63Ag8.653. IgM production by the transformants is compared with Sp603, a subclone of the Sp6 hybridoma.

Selection of IgM(κ)-Positive Transformants. The recombinant plasmid vectors bearing the Ig genes also contain the bacterial gene *neo*, which renders the recipient cells resistant to the antibiotic G418 (17). To transfer the Ig genes into the hybridoma and plasmacytoma cells, bacteria harboring the recombinant plasmids were converted to protoplasts and fused with the various cell lines and G418-resistant cells were selected. Depending on the cell line, the efficiency of G418-resistant colonies ranged between 10^{-4} and 10^{-6} per input hybridoma or plasmacytoma cell (see *Materials and Methods*). The culture supernatant of G418-resistant colonies was tested for TNP-specific IgM by using either a TNP-specific ELISA or by assaying agglutination of TNP-coupled erythrocytes. In various experiments between 15% and 75% of the colonies were positive in such tests.

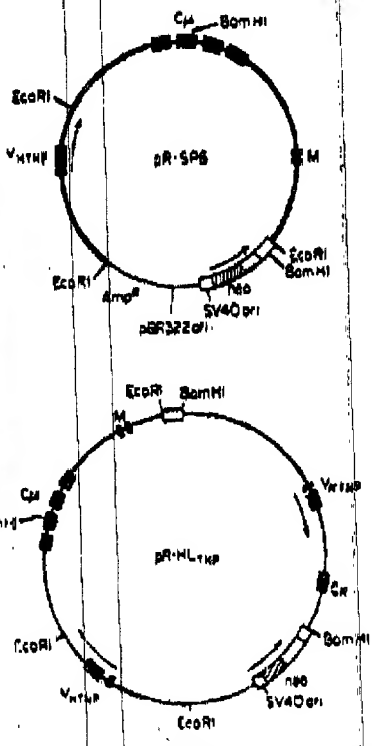

FIG. 1. Structure of the pR-Sp6 and pR-HL_{TNP} plasmids. pR-Sp6 contains the functionally rearranged μ_{TNP} gene (~15 kbp) which was inserted into the EcoRI site of pSV2-neo (see text). In addition to the *neo* gene, pRHL_{TNP} contains the functionally rearranged κ_{TNP} gene (~6 kbp) at the *Bam*HI site (2). Ig genes are represented by heavy dark lines. The directions of transcription of the Ig genes and the SV40 early region are indicated by arrows. The μ and κ exons are shown as filled areas. M denotes alternative COOH-terminal coding regions that are utilized in the synthesis of membrane IgM. Thin lines are of pBR322 origin. Thin white boxes denote DNA derived from SV40, into which the bacterial gene conferring neomycin resistance (hatched box) has been inserted. For specific details concerning the pSV2-neo transfer vector contact D. P. Bergl, see ref. 17.

Analysis of μ_{TNP} and κ_{TNP} Production. Colonies that were positive for TNP-specific IgM were cloned by limiting dilution and examined further. The transformant IR44L1, derived from the κ_{TNP}-positive cell line igm-10 and the μ_{TNP} vector pR-Sp6, makes about 25% of the normal (Sp603) amount of IgM, as measured by the TNP-dependent ELISA. The transformant XR19L4, derived from the cell line X63Ag6 and the μ_{TNP} + κ_{TNP} vector pR-HL_{TNP}, makes about 10% of the normal amount of IgM.

To examine the μ_{TNP} and κ_{TNP} separately, these chains were radiolabeled and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 2). The Sp603 hybridoma cell line still makes the κ chain of its plasmacytoma parent, X63Ag8 (Fig. 2, lane a), as well as the specific μ_{TNP} and κ_{TNP} chains (Fig. 2, lane e). The XR19L4 transformant derived from X63Ag8 has two additional bands (Fig. 2, lane b), which comigrate with the μ_{TNP} and κ_{TNP} of Sp603. The igm-10 cells used here make κ_{TNP} but have ceased to produce the κ of X63Ag8 (Fig. 2, lane c), presumably because of a rearrangement in this κ gene (see legend to Fig. 3). The IR44L1 transformant derived from igm-10 has one new band that comigrates with μ_{TNP} (Fig. 2, lane d). As shown in Fig. 3, analysis of unreduced IgM by $NaDodSO_4$/polyacrylamide gel electrophoresis indicates that the transformants make predominantly pentameric IgM [$(\mu_2\kappa_2)_5$].

RNA Production. To examine the RNAs expressed by the transferred μ_{TNP} and κ_{TNP} genes, cytoplasmic RNA from the transformants was fractionated by gel electrophoresis and probed

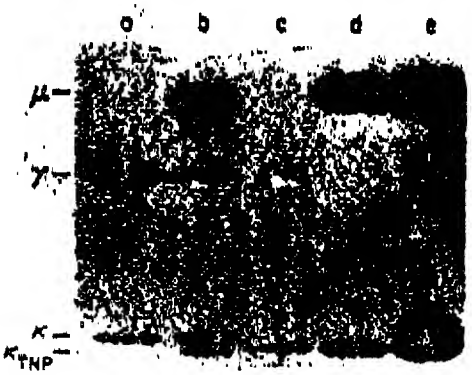

FIG. 2. Analysis of heavy and light chains of secreted Ig. G418-resistant transformant clones were biosynthetically radiolabeled with [^{14}C]leucine as described (7). Secreted immunoglobulins were immunoprecipitated with rabbit anti-mouse IgM antibody complexed with protein A-Sepharose CL-4B beads (Pharmacia). The precipitated material was reduced with 2-mercaptoethanol and analyzed by electrophoresis on a $NaDodSO_4$/polyacrylamide gel. Lane a, X63Ag8; lane b, XR19L4; lane c, igm-10; lane d, IR44L1; and lane e, wild-type hybridoma Sp603.

a b c d e A a b c d e

kb

$(\mu_2\kappa_{TNP_2})_5$

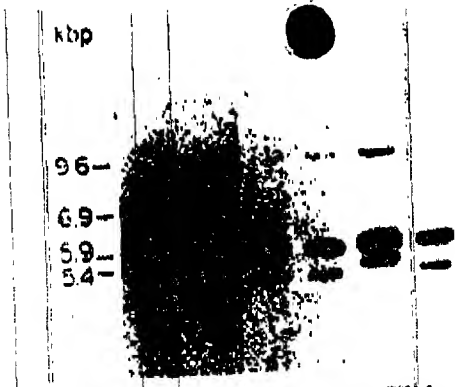

FIG. 5. Detection of pR-Sp6 and pR-HL$_{TNP}$ sequences in DNA from hybridoma cell lines. Lanes a, X63Ag8; lanes b, XR19L4; lanes c, IgM-producing IR44L1; lanes d, Sp6/3; and lanes f, IgM-10 with 6 equivalents of pR-Sp6. *Bam*HI-digested DNA samples (20 μg) were electrophoresed through a 1% agarose gel at 2 V·cm^{-1} for 40 hr and transferred to nitrocellulose. (*A*) A previously hybridized blot (see *B*) was washed according to Thomas (10) and rehybridized to a ^{32}P-labeled probe containing the $C_\gamma 1$ and $C_\mu 2$ exons. This probe was prepared by isolation of the appropriate fragment from a *Xba* I/*Hin*dIII digestion of a genomic clone of the μ-chain constant region gene segment. The bands corresponding to the μ-chain gene-containing fragments generated by *Bam*HI digestion of pR-Sp6 and pR-HL$_{TNP}$ are indicated. The two bands observed in lane e (12 and 14 kbp) correspond to the functionally rearranged μ-gene in the wild-type Sp6/3 cell line. (*B*) The same blot was hybridized with a ^{32}P-labeled probe containing the κ-constant region gene segment that was isolated from the plasmid pL21-5 (donated by R. Wall (22). The bands at 9.6 kb correspond to the κ$_{TNP}$ gene (18). The bands at 6.9, 5.9, and 5.4 kbp correspond to rearranged κ chain genes present in the DNA of the X63Ag8 cell line (23, 24), two of which (6.9 and 5.4 kbp) were retained in the generation of the original Sp6 hybridoma. The 5.4-kbp band corresponds to the functionally rearranged κ gene and this band is not observed in the case of IgM-10 (lane f). Ag8 κ gene and this band is not observed in the case of IgM-10 (lane f). Sizes were estimated by comparison to *Hin*dIII-digested λ phage DNA.

The pattern obtained for XR19L4 upon hybridization of the same blot with the C_γ probe is consistent with the above interpretation. DNA from this transformant contains a 9.6-kbp fragment corresponding to the wild-type κ$_{TNP}$ gene (18) in addition to other fragments that correspond to the κ chain genes endogenous to the recipient X63Ag8 cell line (23, 24).

Assay of IgM Function. We have tested the normal functioning of the IgM produced by the transformants by assaying its action in complement-dependent lysis of TNP-coupled erythrocytes (Table 1). The IgM concentration in the culture supernatants of the indicated cell lines was measured by the hemolysis of protein A-coupled erythrocytes in the presence of anti-IgM (7). These results indicate that IgM made by IR44L1 has normal activity with regard to TNP binding and complement activation. However, the transformant XR19L4 makes IgM that has an activity that is less than 1/30th of the normal activity in the TNP-dependent hemolysis assay. X63Ag8 still produces the myeloma κ chain, and this κ chain can be incorporated into IgM, thus reducing TNP-specific hemolysis activity (7). To avoid this problem of the nonspecific myeloma κ chain, the μ$_{TNP}$ + κ$_{TNP}$ vector pR-HL$_{TNP}$ was transferred into the nonproducer cell lines Sp2/0Ag14 (5) and X63Ag8.653 (4). The IgM produced by transformants of these cell lines has normal activity for TNP-specific hemolysis (Table 1).

DISCUSSION

We and others have previously reported the expression of Ig light chain genes in various cell types (2, 26–29). In this paper we have described the construction of plasmids that bear genes for TNP-specific immunoglobulin μ and κ chains. The expression of these genes was studied after the transfer of the plasmids into various cell lines derived from Ig-secreting plasmacytomas or hybridomas. The transfer of these plasmids into these cells is usually (see below) sufficient to cause the production of pentameric IgM(κ) that binds antigen (TNP) and activates complement—that is, these cell lines (X63Ag8, X63Ag8.653, IgM-10, and Sp2/0Ag14) provide all of the machinery necessary for IgM production except the structural genes for the μ and κ

chains. The capacity to provide this machinery is present despite the fact that these cell lines have been propagated for years without overt selection for this property.

We expect that this system will be very useful in determining the structural requirements for normal IgM production and function. To date, the use of genetics for this purpose has been limited to the analysis of naturally occurring mutants that interfere with normal IgM processing and activity (7, 30). Although such mutants are useful as a starting point, *in vitro* mutagenesis offers a more rapid and systematic method of obtaining altered IgM. Thus, it should be possible to identify the amino acids that are critical for complement activation or Fc receptor binding. Similarly, one can expect to define the features that are necessary for pentamer formation, glycosylation, and secretion.

As is the case with other gene-transfer systems, we have found that the various transformants produce quite different amounts of μ and κ chain, ranging from undetectable to approximately normal levels. In general, a linear relationship does not exist between the copy number of the transferred sequences and the

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18. Gough, N. M., Kemp, D. J., Tyler, B. M., Adams, J. M. & Cory, S. (1980) *Proc. Natl. Acad. Sci. USA* 77, 554–558.

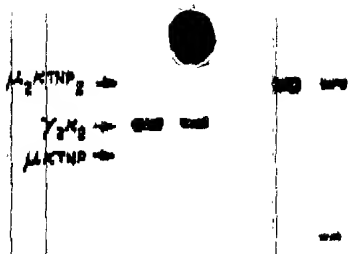

FIG. 3. Analysis of secreted (unreduced) Ig. The radiolabeled culture supernatants as described in the legend to Fig. 2 were analyzed by electrophoresis on a $NaDodSO_4$/polyacrylamide gel without reducing the disulfide bonds (7). Lane a, X63Ag8; lane b, XR19L4; lane c, igm-10; lane d, IR44L1; and lane e, wild-type hybridoma Sp603. The markers indicate the major forms of Sp603 IgM and X63Ag8 IgG1.

with various μ- and κ-specific DNA sequences (Fig. 4). RNA for the μ heavy chain was detected with a probe from the $C_\mu 4$ region. The transformants XR19L4 and IR44L1 have bands at both 2.7 and 2.4 kilobases (kb), whereas the parental hybridoma Sp603 has only one band at 2.4 kb (Fig. 4A). A genomic probe containing the μ membrane-specific exon hybridized only to the 2.7-kb band (data not shown). RNAs of 2.7 and 2.4 kb have been found to encode the membrane (μ_m) and secreted (μ_s) forms of the μ chain, respectively (19–21). These results suggest that, whereas Sp603 makes RNA only for the μ_s form, the transformants make RNAs for both μ_m and μ_s. However, we have been unable to detect membrane IgM by staining with fluorescent μ-specific antibodies. The μ_m form has a longer polypeptide chain than does the μ_s form and consequently can be distinguished from μ_s by its lower mobility in $NaDodSO_4$/polyacrylamide gel electrophoresis. Therefore, we examined intracellular μ chains that were biosynthetically radiolabeled in the presence of tunicamycin; for each transformant we found only one μ band, and this band comigrated with the μ band of Sp6 (results not shown). These observations suggest that either the 2.7-kb RNA is not translated or that the μ_m protein is very short-lived in the transformants.

In a similar manner, the RNA blots were hybridized with a probe derived from the κ_{TNP} V region. Compared to Sp603 and igm-10, the transformant XR19L4 was found to make a low amount of a 1.2-kb RNA that comigrated with authentic κ_{TNP} RNA (Fig. 4B).

Structure of Transfected DNA. To analyze the organization of the transferred pR-Sp6 and pR-HL$_{TNP}$ plasmids in the transformed cell lines, *Bam*HI-digested cell DNA was hybridized with probes specific for the μ- and κ-chain constant region gene segments. The $C_\mu 1$–2 probe used here spans the *Bam*HI restriction site in the $C_\mu 2$ exon (Fig. 1). Therefore, a minimum of two fragments is expected to be detected with this probe.

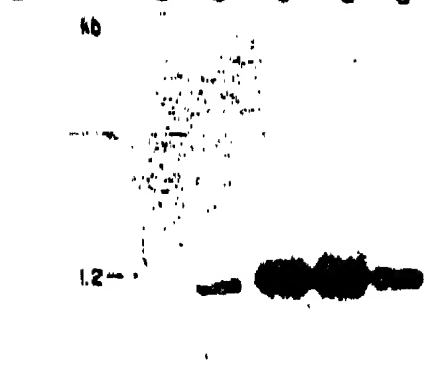

FIG. 4. Detection of μ_{TNP} and κ_{TNP} gene sequences in cytoplasmic RNA from transformed cell lines. Lanes a, X63Ag8; lanes b, XR19L4; lanes c, igm-10; lanes d, IR44L1; and lanes e, Sp603. Ten micrograms of total cytoplasmic RNA (8) was denatured with glyoxal, electrophoresed through a horizontal 1% agarose gel in 10 mM sodium phosphate buffer at pH 6.8, and transferred to nitrocellulose as described by Thomas (10). (*A*) The blot was hybridized with a ^{32}P-labeled probe corresponding to the $C_\mu 4$ exon. This probe was isolated from the cDNA clone pH76μ17 (donated by J. Adams) after digestion with *Pst* I (18). (*B*) A similar blot was hybridized with a ^{32}P-labeled probe containing κ_{TNP} V-region coding sequences (16). Sizes were estimated by comparison to mouse ribosomal 28S and 18S RNA (4.7 and 2.0 kb, respectively).

Two fragments of 6.0 and 16 kbp were detected in the DNA of both of the transformants. These correspond to the fragments generated by *Bam*HI digestion of the intact pR-Sp6 and pR-HL$_{TNP}$ plasmids (Fig. 5). In addition, one (XR19L4) or two (IR44L1) extra fragments could be detected in the DNA from these cell lines. In parallel experiments, sequences indicative of unintegrated pR-TNT plasmids have not been detected in the low molecular weight fraction of the Hirt supernatants (25) of similarly transformed *igk-14* cells (results not shown). Taken together, these results suggest that the transferred genes are tandemly integrated into the chromosomal DNA of the recipient cells.

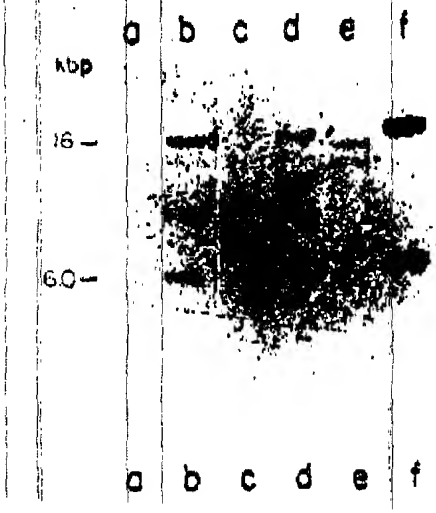

Table 1. Assay of functional IgM

Cell line	Phenotype	Hemolysis titer on erythrocytes coupled with: Protein A	TNP	TNP/protein A ratio
Sp603	IgM, κTNP + κX63	2^6	2^8	4
igm-10	κTNP	<1	<1	—
IR44L1		2^2	2^4	4
X63Ag8	IgG1, κ	<1	<1	—
XR19L4		2^6	<1	<1:8
Sp2/0Ag14	No Ig	<1	<1	—
SR1.2		2^4	2^6	4
SR40.1		8	2^2	2
X63Ag8.653	No Ig	<1	<1	—
X653R1.1		2^4	2^6	4

As described in the text, the transformants IR44L1 and XR19L4 were derived by introducing the μ_{TNP} gene alone or the μ_{TNP} and κ_{TNP} genes together into the igm-10 and X63Ag8 cell lines. Similarly, the cell lines SR1.2, SR40.1, and X653R1.1 were generated by transferring the μ_{TNP} + κ_{TNP} vector pR-HL$_{TNP}$ into Sp2/0Ag14 and X63Ag8.653. The indicated cell lines were grown to approximately 10^6 cells per ml, and cul-

Exhibit B

PROPOSAL TO BECTON-DICKINSON

Leonard A. Herzenberg
Department of Genetics

TRANSFECTION OF CHIMERIC IMMUNOGLOBULIN GENES INTO LYMPHOID CELLS

TRANSFECTION OF CHIMERIC IMMUNOGLOBULIN GENES INTO LYMPHOID CELLS

Vernon T. Oi and L.A.Herzenberg [redacted]

The objective of this project is to transfect two chimeric immunoglobulin genes into a lymphoid tissue culture cell line capable of transcribing and translating these genes into proteins. The chimeric immunoglobulin genes will be constructed using standard recombinant DNA techniques and will consist of (1) a V-D-J gene segment coding for a dansyl hapten binding V-region and a Igh-b allotype constant region; and (2) a mouse V-D-J gene segment coding for a human cell surface antigen (e.g., Leu-2) and a human immunoglobulin constant region.

THE METHOD TO DELIVER DNA INTO THE CELL. There are currently five techniques being used to transfect DNA into eukaryotic cells. All five will be examined as possible means to introduce active immunoglobulin genes into lymphoid cells. The techniques include: (1) Ca-PO4 precipitation; (2) PEG 6000 fusion of lambda phage particles; (3) vesicle fusion; (4) protoplast fusion; and (5) microinjection.

THE APPROPRIATE DELIVERY VECTOR. We have available to us suitable first generation SV40-pBR322 vectors to contain the recombinant immunoglobulin genes to be used in transfection experiments. Further development of these vectors also will be undertaken.

THE APPROPRIATE CELL HOST. Since the chimeric SV40-pBR322 vectors we are planning to use contain either the thymidine kinase or guanine phosphoribosyl transferase genes as selectable eukaryotic markers, we intend to develop lymphoid cell lines that lack these enzymes to use as transfectant recipients. These cell lines must have the potential to express immunoglobulin genes, but lack the ability to produce endogenous immunoglobulin products.

RECOMBINANT DNA. Standard recombinant DNA techniques will be used to isolate a DNS V-D-J gene segment from the genome of an existing hybridoma cell line producing anti-DNS antibodies. Igh-b constant region genes, as well as human constant region sequences will be isolated similarly. Chimeric recombinant V-D-J-Constant region sequences will be constructed from these newly isolated gene segments.

SELECTION OF TRANSFECTED CELL LINES EXPRESSING NOVEL IMMUNOGLOBULIN GENES. Should all of the above be accomplished, successfully transfected cell lines will be selected by enzyme markers (TK and GPT) and with the fluorescence-activated cell sorter using techniques and antibody reagents already developed.

Exhibit C

APPLICATION FOR A RESEARCH OR CLINICAL INVESTIGATION GRANT

(Please read carefully the attached "Policies on Research and Clinical Investigation Grants" and the instructions on all pages of this form, before completing this application.)

TO: American Cancer Society, Inc.
777 Third Avenue
New York, New York 10017

Date [redacted]

Application is hereby made for a grant* in the amount of $ [redacted]
for the period from [redacted] to [redacted] inclusive.

Title Expression of Transfected Mouse and Human-Mouse Hybrid Immunoglobuli

FROM:

Sherie L. Morrison, Ph.D. — Sherie L. Morrison
Name of Investigator — Signature

Associate Professor of Microbiology — (212) 694-4183
Title — Telephone No.

Microbiology — College of Physicians and Surgeons
Department — Division of Institution

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*It is understood that each applicant, by the act of applying for a grant, agrees, if the grant is made, to abide by the Society's POLICIES ON RESEARCH AND CLINICAL INVESTIGATION GRANTS.

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APPLICATION FOR A RESEARCH OR CLINICAL INVESTIGATION GRANT

SUMMARY OF RESEARCH PROPOSED

Name and Official Title of Principal Investigator

Dr. Sherie L. Morrison, Associate Professor of Microbiology

Name and Address of Applicant Organization

Columbia University College of Physicians and Surgeons,
701 West 168th Street, New York, New York 10032

Title of Project

Expression of Transfected Mouse and Human-Mouse Hybrid Immunoglobulins

Use this space to summarize concisely your proposed research. Outline objectives and methods. Underscore the Key words (not to exceed 10) in your abstract.

Gene transfection has become an increasingly popular method of studying gene expression. We have recently developed methods of transfecting immunoglobulin genes into myeloma cell lines; these genes are efficiently expressed. The current experiments will define the regions of the mouse heavy and light chain genes which are required for efficient transfection and those required for high level immunoglobulin expression. Once these sequences are defined we will determine the influence of their position in the molecule on their function. We also will construct novel molecules and study their expression and function. In particular we will determine if hybrid molecules with the variable region from a mouse immunoglobulin (Ig) fused to the constant region of a human Ig molecule can be effectively produced and function. Secondly, we will examine the expression and function of molecules made from gene fragment. We will see if light chain dimers, one light chain of which has a heavy chain variable region can bind antigen. Such hybrid molecules have potential therapeutic value in treating human diseases such as cancer.

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2. Aim and Method of Study:

A. Specific Aims:

The aim of these studies is to produce novel immunoglobulin (Ig) molecules by using DNA mediated transformation of myeloma cells. The project will proceed in several steps.

a. Initially we will develop an optimum transfection system and define the regions of the mouse kappa light chain gene which are important for increased transformation frequencies. We will also investigate if other Ig genes contain sequences of similar function and attempt to define the mechanism leading to the increased transformation frequency.

b. Secondly, we will define the regions of both heavy and light chain genes which are required for efficient expression. Such a definition is required to permit the rational assembly of novel molecules which will be produced at high levels.

c. Thirdly, we will produce hybrid human-mouse Ig genes, test for their efficient synthesis in transfected cell lines, and assay the biologic activity of the novel molecules. We will also attempt to produce variant proteins of altered structure and function.

B. Methods

1. Immunoglobulin Genes To Be Used

In the initial studies we will use the heavy and light chain genes from the S107 myeloma. Genomic clones of both of these expressed genes have been obtained from Dr. Matthew Scharff and are available in the laboratory. Initial expression studies (see above) have focused on the S107A kappa chain gene. We will now also construct a vector containing the S107 heavy chain gene so that we can study its expression. To date three human heavy chain genes have been acquired. A VDJ segment and the $\gamma 1$ gene have been acquired from Dr. Honjo and a kappa chain gene from P. Leder. We will initially study the expression and function of mouse V_L-human C_κ and mouse V_H-human $C_{\gamma 1}$ constructs.

2. Recipient Lymphoid Cell Lines

Our principal recipient cell line will be the mouse myeloma J558L. This produces a λ light chain, no heavy chains and transfects very well. Because λ and κ are so different structurally we anticipate little competition between these molecules in assembly with heavy chain. However, if the λ chain presents a problem we will isolate a non-producing variant of J558 using methodology which is routine in the laboratory.

3. Sequences Necessary for Efficient Transformation.

The S107A light chain gene is contained on a 7 Kb Bam H1 fragment as diagrammed in Figure 2. The deletions and partial molecules shown in Figure 2 have already been constructed and are being assayed for their transfection efficiency. Using the sites shown in the figure and others which we identify we will further assay the gene for transfection enhancement. The general protocol will be to subdivide the gene into fractions and assay each for its influence on transformation frequency. In particular we will put the Bam-Bgl or Bgl-Bgl pieces from the 7Kb L chain fragment into the Bam site of pSV2gpt and assay for transfection frequency. Other small fragments will be excised, blunt ended and Eco R1 or Bam H1 linkers put on. Bam H1 linkers have already been put on all the Hae III pieces from the L chain gene. Each fragment will be assayed for its enhancement of transformation; combinations of fragments will also be assayed to determine either synergistic or antagonistic interactions. Positive fragments will be subdivided into smaller pieces either by cutting with additional restriction enzymes or by cutting with progressive exonucleases such as Bal 31. The general objective will be to localize to as small a region as possible active sequences. The nucleic acid sequence of such regions will be determined and homologies between active regions sought.

Several possible mechanisms can be proposed to explain the increased transfection frequency: 1) replication of the plasmid as an episome; 2) increased expression of the selectible gene, in these experiments XGPT, or 3) increased integration into chromosomal DNA. We will try to distinguish among these possibilities.

Replication as an episome could be either transient during the early stages of the transfection or persistent. Transient replication increases the copy number of the plasmid within the cell and hence the probability of productive integration. To test for transient expression as an episome, 72 hours after transfection the Hirt supernatant (23) will be prepared from the transfected cell lines and the small molecular weight DNA examined by Southern (24, 25) blot after cleavage with the restriction endonuclease Mbo 1, and if available, Dpn I. Both Dpn I and Mbo 1 recognize the sequence GATC. If unmethylated this sequence is cut by Mbo I but not Dpn I; the sequence $G^{me}ATC$ is cut by Dpn I but not Mbo I. Since the dam methylase of E. coli introduces methyl groups on the N^6 position of adenine in the sequence GATC, while no eucaryotic enzymes do, it is possible to distinguish between DNA replicated in bacteria and that replicated in mammalian cells by the methylation pattern. To test for persistance as an episome, the Hirt supernatant will be isolated from stable transformants. Southern blot analysis will be done both on uncut DNA to test for the occurrence of DNA in the supercoil form and cut with restriction enzymes to assay for restriction fragments of the appropriate size. In addition, material from the Hirt supernatant will be used to transform bacteria. If replicating plasmids

are present they should be effective in transforming bacteria. If transformed bacteria are obtained, plasmid DNA will be isolated from them and the nature of the plasmid DNA determined following digestion with restriction endonucleases. In previous studies using these types of vectors, episomal replication has only been detected in Cos cells where T antigen is supplied in trans (26).

Analysis of stable transformants has already shown that the amount of gpt produced in those transfected with a pSV2gpt-S10721 is not consistently different from that produced in cells transformed using pSV2-gpt. However that does not exclude the possibility that increased transient expression of XGPT may lead to increased transformation. To test that possibility cytoplasmic extracts prepared from cells 48-72 hours after transfection will be assayed for their XGPT activity (9 and appended reprint). In the vector which we have routinely used for transfection, the SV40 early promoter has been used to drive the bacterial XGPT gene. It is also possible to use the promoter from the Herpes thymidine kinase gene (appended manuscript) to drive the XGPT gene. We will assess if sequences effective in enhancing transfection by vectors using the SV40 promoter are effective with the TK promoter and if these sequences lead to increased transient expression of sequences off the TK promoter.

It is also possible that increased transformation results from an increased frequency of vector integration into chromosomal DNA. It is difficult to directly test this hypothesis. However we will do Southern blot analysis following cleavage with restriction endonucleases with 6 base recognition sequences of DNA isolated from transformants obtained using vectors either with or lacking the enhancing sequences. This analysis will give us an estimate of the number of sites of integration per transformant. If the same size restriction fragments are found in independent transformants it will suggest a common site of integration. To confirm this it would be necessary to clone the integrated genes and directly analyze the flanking sequences. Methods to produce genomic libraries using lambda phages are available in the laboratory.

4. Identificaion of Genetic Sequences Necessary for High Level Immunoglobulin Expression.

Preliminary experiments have demonstrated that it is possible to introduce a rearranged mouse kappa light chain gene back into a mouse myeloma cell by DNA mediated transformation; the reintroduced light chain can be expressed within the myeloma cell to levels approaching that of the endogeneous myeloma light chain. Deletion analysis has also suggested that sequences within the IVS are required for efficient Ig expression. By cutting with Hind III we can now mix and match the 5' and 3' deletions. We will do these experiments to precisely define the extent of the region necessary for expression. Once we have appropriately located the sequences, we will make additional Bal 31 deletions to try and locate the sequences to within one or several

nucleotides. The end points of the deletion will be sequenced and compared to the published sequence of the IVS (14) to accurately position them.

Once the IVS necessary for high level Ig production has been accurately identified we will do further analysis of the effects of this sequence and the structural requirements for its function. We will determine if there is a position effect on Ig production, that is, must the sequence always be at the same position and in the same orientation in the Ig gene to exert its enhancing effect. The SV40 enhancers provide an example of an enhancer that functions in various positions and orientations. The Ig sequences which failitate Ig production will be placed both 5' and 3' of their normal positions in the Ig gene and elsewhere in the expression vector in either orientation and the level of Ig expression assayed. Linkers will be put on the active fragment. By using linkers, we will invert the sequence in its normal site, and also duplicate it in both its normal and inverted orientation. Random small insertions (21) will also be put into the active sequence to define its structural requirements for function. We will make constructions with IVS consisting only of the required sequence and enough information to preserve the 5' and 3' splice junctions. In addition we will determine if the Ig sequences increase the expression of genes being synthesized off non-Ig promoters. Vectors exist with the bacterial XGPT gene being expressed using either the SV40 or the Herpes thymidine kinase (TK) promoter. The Ig sequences will be placed at various positions relative to the SV40 and TK promoters and the synthesis of XGPT assayed both in transient expression experiments and in stable transformants.

We will also test for the influence on expression of sequences 3' to the coding region. We have available a kappa cDNA clone with R1 ends. We will convert these R1 ends to BAM ends by blunt ending with S1 or T4 polymerase and adding BAM·linkers. We will then exchange the 3' Hpa 1-Bam fragment from the cDNA for the same fragment from the pSV2-S107-21 vector. The resulting vector will lack sequences 3' to the mRNA. If this light chain is efficiently expressed we will do Bal 31 digestion before putting on the Bam linkers. Exchange of the Hpa-Bam fragments after Bal 31 digestion will delineate how much of the 3' sequence is required and if it is necessary to have a poly A addition site. We can add back a poly A site from SV40 to provide a new poly A site at a different position.

The sequences 5' to the gene necessary for expression will also be determined. Preliminary construction will be done by cutting with R1 + Pvu II and R1 + partial Xba, putting on R1 linkers, reclosing and assaying. Bal 31 digestion can be done before putting on the linkers to more accurately define the required sequences. The present experiments will be designed merely to identify the extent of the necessary sequences. Fine structure mapping of the promoter sequences by such methods as in vitro mutagenesis and "linker scanning" (21) are beyond the scope of the present proposal.

The experiments detailed about all relate to expression of the kappa chain gene. A similar series of experiments will be done to identify IVS, 5' and 3' sequences necessary for expression of the S107 H chain gene. For H chains we will also determine if the synthesis of a light chain, either specific or non-specific, is required for or facilitates expression.

To assay for the synthesis of the transfected gene product cells will be labeled with ^{14}C-valine, threonine, and leucine, cytoplasmic extracts made (27) and the Ig immunoprecipitated. Specific immunoprecipitable chains will be identified using SDS gels. We have found that the S107 kappa chain can easily be separated from the J558 lambda chain using SDS-PO_4^- gels (unpublished results). In selected experiments 2-D gels also will be used to identify the products of transfected genes (7).

The amount of the transfected product synthesized will be quantitated in two ways. Firstly, the ratio of the amount of synthesis of the endogeneous immunoglobulin light chain to the transfected light chain will be determined by scanning the autoradiograms of SDS gels of immunoprecipitates from transfected cells. If labeling is done for a short period of time so that neither chain is secreted or significantly degraded this method gives a good estimate of the relative rates of synthesis. To quantitate the synthesis as a percentage of the total protein synthesis, cells will be labeled for short periods of time (3-5 minutes) with ^{14}C-amino acids, the total amount of TCA precipitable material synthesized determined, and the amount of TCA precipitable material which is immunoprecipitated determined. Pulse chase experiments will be used to determine the rate of degradation of the immunoglobulin. Long term (3-24 hours) labeling with ^{14}C-valine, threonine, and leucine, immunoprecipitation and SDS gel analysis of the secreted product (with and without reduction) will determine what product is secreted and whether it is assembled.

Northern blot analysis and hybridization with Ig specific ^{32}P-labeled probes will be used to determine the approximate size and heterogeneity of any Ig specific transcripts in the cell lines. Formaldehyde gels and the blotting procedure of Thomas (28) is used routinely. In the cases where the recipient cell line synthesizes an immunoglobulin with the same constant region as the transfected gene, variable region probes will be used.

The 5' and 3' end of the cytoplasmic transcripts and points of splicing of the IVS will be mapped using the S1 nuclease resistance method of Berk and Sharp (29). In the case of the S107A gene the plasmid will be labeled at the Hpa I site in the constant region using T4 polymerase and the 1.5 Kb Hpa I to BAM H1 fragment used to identify the 3' end of the transcript. Label of the Hpa I site with kinase will be used to position the 3' end of the IVS and label of the Kpn site within V with T4 polymerase will be used to locate the 5' side of the IVS.

Because there is an IVS between the leader sequence and V_L and no known unique restriction site in the leader sequence, templates synthesized in M13 will be used to map the 5' end of the transcripts. Hind III linkers have already been attached to the Hae III fragment which contains the region 5' to the light chain gene and the 5' end of the variable region and should contain the light chain promoter region. This fragment will be cloned into M13, and used to synthesize message complementary probe for S1 mapping experiments. If some transcripts originate 5' of this fragment, a larger fragment will be cloned into M13. S1 analysis will be done on RNA isolated from both the transient expression experiment and from stable transformants. We have already used S1 analysis to demonstrate that the 3' ends of the mRNA from transient expression and stable transformants with many of the vectors are identical.

The Northern blot and S1 analysis will yield information about the structure of steady-state cytoplasmic mRNA. To gain some information about nuclear RNA, it will be isolated from selected transformants and the size of the nuclear transcripts determined by Northern blot analysis. Initial blotting will be done with probes which contain the entire Ig gene. Region specific probes will be used to both elucidate the pattern of processing and to identify abnormal transcripts. A necessary control for these experiments will be a careful analysis of the nuclear RNA of the recipient cell lines to eliminate the possibility that they contain aberrant transcripts of Ig genes.

5. Expression and Function of Novel Immunoglobulin Molecules

Once we have a clear idea of the sequences necessary for efficient Ig production we will begin to construct novel Ig molecules and will study their expression and function. Combinations which we will produce include:

a. [S107 kappa] + [S107 alpha]
b. [V_H S107 + γ1 human] + [S107 kappa]
c. [V_L S107 + κ human] + [S107 alpha]
d. [V_H S107 + γ1 human] + [V_L S107 + κ human]

In these constructions both the H and L chain will be covalently linked into the expression vector to increase the probability of their cotransformation and expression.

Combination a will demonstrate that it is feasible to establish an antigen binding cell line by gene transfection. Combinations b and c will demonstrate whether it is possible to get expression of hybrid molecules, and if it is possible to assemble molecules, one constant region of which is of murine origin, the other of which is human. Combination d will demonstrate if it is possible to express a molecule with the specificity of murine origin, but the constant region and effector functions of human origin.

If we achieve efficient expression using the entire gene we will make and analyze a series using only gene fragments. Among the combinations which we plan are:

a. $[V_L$ S107 + C_κ human$]$ + $[V_H$ S107 + $\gamma 1$ human with CH_1 deletion]
CH_2 deletion]
CH_3 deletion]
$CH_2 + CH_3$ deletion]
$CH_1 + CH_2$ deletion]
$CH_1 + CH_3$ deletion]

b. $[V_L$ S107 + C_κ mouse$]$ + $[V_H$ S107 + C_κ mouse$]$

c. $[V_L$ S107 + C_κ human$]$ + $[V_H$ S107 + C_κ human$]$

All transformants will be assayed, using the methods detailed above for the synthesis, assembly and secretion of Ig molecules. Transcripts will be analyzed both for their fidelity and quantity.

One of the reasons for using the S107 V_H and V_L is that they come from a molecule of known antigen specificity, an anti-phosphorylcholine antibody. Recombinant molecules will therefore be assayed for their ability to bind phosphorycholine (PC). This can efficiently be done by labeling the proteins by growing the cells in ^{14}C-VTL and then testing for binding to PC-Sepharose. The proteins binding will be analyzed on SDS gels following elution. Human $\gamma 1$ fixes complement. If recombinant molecules bind antigen, their ability to fix complement will be tested. Resistance to serum protein proteases will be tested by incubating biosynthetically labeled proteins in serum at 37°C for varying lengths of time, and then analyzing the amount of Ig which can be immunoprecipitated. Immunoprecipitated material will be run on SDS gels to determine its size. Serum half-life will be tested by injecting biosynthetically labeled proteins into mice and following their serum decay. It would be desirable to assess these parameters in humans, but such experiments are beyond the scope of this grant.

Exhibit D

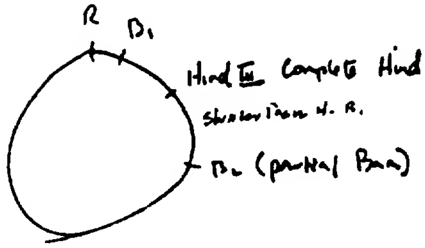
[redacted]

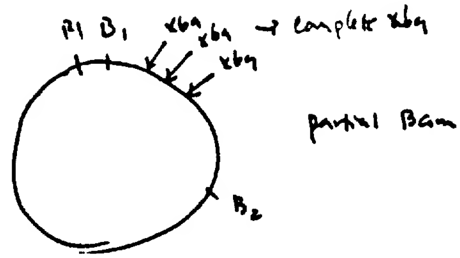
pSV2dH-S107 Hinc

Hinc Vector   Hinc

Xba-Bam Vector   MPC-11 Kappa

pSV2dH S107





① Linear Hind
② Big cut B2 *
③ cut B1
④ cut B1 and B2
⑤ small H-B2
⑥ small H-B1

① Xba-linear
② Big-cut B1
③ cut B2
④ cut B1 and B2
⑤ small fragments B1-Xba Xba Xba B2

Hinc in charon 28

4.5-Hind-Hind → should clone legible & leading to compatible ligation
4.85 Hind-Bam *

Arms: 22.8kb
9.2kb

MPC-11 Kappa.
Vector 4.4kb. Bam-Bam
4.4kb * Bam Xba
4.2kb. Xba Bam

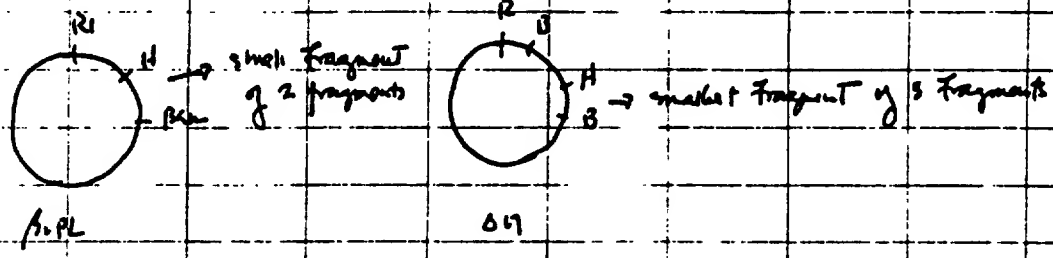
There are additional ~~Bam~~ Xba sites in this kappa light chain gene

[redacted]

pUC minus homologous control vector

Rx — Hind III — Bam → linear pUC

Replace Hind III-Bam fragment with Hind-Bam from Δ17 vector



2ml DNA	2ml DNA
5ml Rx mix	5ml Rx mix
2.5 + 2.5 Loading mix	2.5 + 2.5 Loading mix
35ml H_2O	35ml H_2O

This digestion looked incomplete; and the order of loading samples onto the gel was reversed.

∴ Take precautions in analysing these this plasmid construction.

This is further complicated by the fact that this tube was labelled pSV2neo-S107 → confirm with the actual pSV2neo-S107

42 383

[redacted]

pSV2neo-βcPL

pSV5-neo

Neo βcPL

Neo is cut with Bam-R1

Largest fragment is the vector host

βcPL - cut with R1 - Bam

insert excised and should be - this isn't true?

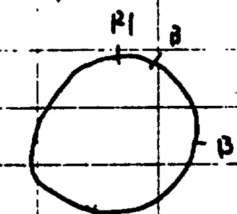
larger than vector host

Neo S107

Neo vector is the same as ←.

S107 - cut with R1 to completion

Bam - partial



② Bam Bam = 7

① R1-Bam Bam 7.3

③ Vector

④ R1-Bam "filler"

It appears this S107 did not cut at all; either because it received no enzyme or because there is an inhibitor

[redacted]

Neo 9.2ml = 3ug	pSV2βH-S07K 11ml = 4ug.
5ml R1 salts	5ml R1 salts
2.5x1.5 enzymes	3.0ml R1 enzyme
30ml H2O	30ml H2O

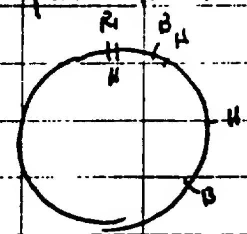
[redacted]

MPC-11 kappa

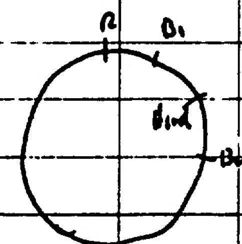
pSV2gH-MPC11K

complete Bam cut

partial Hind III



pSV2gH-S107-21K



(R1 salts) Bam complete → 2 bands

8.4 ① ⑤ 4.4

Bam-Hind ~7.9 ② ⑥ 4.1 (with Hind cut)

* Bam-Hind ~5.4 ③ ④ 0.3

Hind Hind ~4.9 ⑥

Hind Bam ~3.7 ⑦

Bam-Hind - 0.5 ⑧

DNA = 1mg/ml 3ug = 3ul

37ul H2O

5ul R1 salts

[3.5 x 25] enzymes

Hind III complete   Hind salts [addition of Bam salts]

partial Bam

① Linear Hind

② cut B2

③ cut B1 *

④ cut B1+B2

⑤ Small H-B2

⑥ small H-B1

354ug/ml 5ug

3.5ug = 9.9ul

Hind

5ul R1 salts

add 3ul Hind enzyme.

with salt 0.9 Bam → partial 5 min.

addn. 2ul Bam

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Mapping Experiment [redacted]

				ug volume	4 ug/tube RI units	ug RI with StuI	Hind III units FnuBII
1	5	pSV2ΔH gpT	610 ug/ml	1.6 ul			
2	6	pSV2ΔH-S107-24	354	2.8			
3	7	pSV2ΔH-MPC-K	350	2.9			
4	8	pSV2 ΔH S107 Hnk	260	3.8			

9 cut c RI

2.9ul = 1 ug 5ug = 2.9×5 = 14 ul

100 ul volume − 10 ul RI units − 14 ul DNA = 76 ul (−5.25 ul = 71 ul new volume)

14×3 = 42 units 5.25 ul of StuI

Strategy: 1. Linearize plasmid @ StuI site

2a. Link with Hind III linkers → when linkers are cut, an internal deletion will be generated

2b. Link with Xho I linkers → link the 3' end of the conserved sequence with Xho.

5' promoter element analyses of MPC-11K V5 gene. PvuII partial cut

PvuII − 1500 U/188 ul: 8 u/

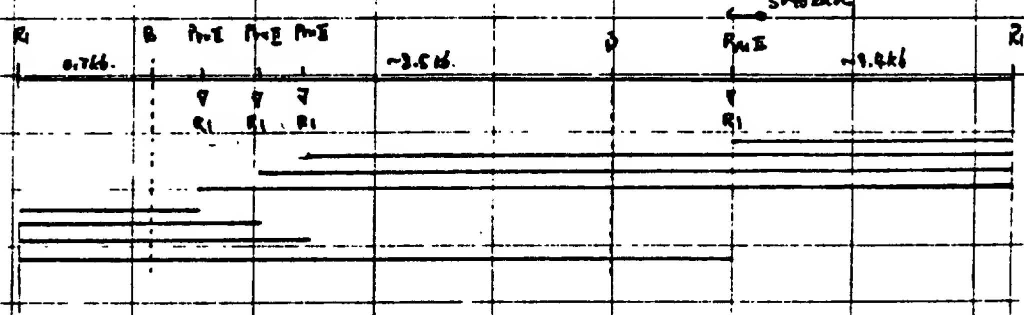
20 ug MPC-11K

20×2.9 = 58 ul

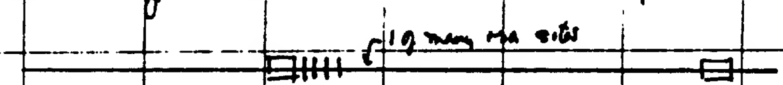
200 − 20 (salts) − 58 −

3 ul (Enz) = 119.5 ul

H2O (Stop = EDTA



Xho linker into RsaI site of MPC-11K. partial RsaI → link with XhoI linkers.



Strategy: ① partial cut ② Xho linker ③ Isolate largest fragment for full unit length?

5 ug MPC-11K → 5×2.9 = 14.5 ul (Hind III salts). 1 ul of RsaI = 10 units → 6 min digest

Exhibit E

TO

Subject

[redacted] VTO started bulk O.N. E.4 + amp

[redacted] ICE.18
PSV2ΔH S107 VHCKΔ17.8
PSV2ΔH S107 VHCK 7
HUG.2
– process

– 18 20mls – .8 m NaCl
7 20 "
2 20 "
17.8 20 " lots of precip
–20 ON.

[redacted] spin, resusp + RNase 10:30am
spin 49K 2 days

[redacted] – harvest ICE 18 small band

		H_2O	EtOH
ICE 18	.8 mls	1.6	4.8 mls
HUG 2	1.0 mls	2.0	6.0
VHCK17.8	.9 mls	1.8	5.4
VHCK 7	1.0 mls	2.0	6 mls

– –20° O.N.

[redacted] – spin + wash 2 5 mls EtOH + spin + lypho.
– resusp

		260	280	320		for Bud + [illegible]	
ICE 18	1.5 mls TE	.149	.072	.001	745 µg/ml.	R 2.06	1118 µg
HUG 2	2.0 mls	.235	.119	.005	1175 "	R1.97	2350
VHCK17.8	2.0 mls	.173	.084	.001	865 "	R 2.05	1730
VHCK 7	2.0 mls	.168	.087	.001	840 "	R1.93	1680

Signed .... Date ....

[redacted] freeze 2.5mls O.N. + .18mls DMSO + .5mls aliquot

- process maxi minis from 20mls O.N.

HUG1 Sal
HUG2 Sal
VHCK 7 Sal
ICE Bam

- spin 5K 5mins 15mls (HB.4)
- resusp 1ml gluc. /lypo /sample
- 10mins R.T.
- add 2mls NaOH/SDS & mix by inverting
- 10mins on ice
- add 2mls pot. acetate /sample + mix
- 10mins on ice.
- spin 7.5K 10mins HB.4
- 4mls phenol /chloro. + mix & spin
  2mls " " "
- 8mls EtOH + 5mins R.T.
- spin 7.5K 10mins HB.4
- wash C R.T. 70% EtOH
- spin 7.5K 10mins
- dry briefly
- resusp 600 ul TE + 1.6ul RNase

digest 3:30pm 37°

1 HUG1 } 30ul DNA
✓5ul RI salts
2 HUG2 } 2.0ul RI
2.5ul Sal I
✓10.5 ul H_2O

3 VHCK-7 30ul DNA
✓5ul RI salt
2.0ul RI
2.5ul Sal I
✓10.5ul H_2O

4-8 VHCK7 30ul DNA
✓5ul RI salts
2.5ul Sal I
✓12.5ul H_2O

Signed.................. Date..................

Subject 2

[redacted] start 20ml O.N. from plates [redacted]

HUG 1 Sal +amp LB
HUG 2 Sal " } 37° shaker
VHCK7 Sal "
ICE. Bam 25ul CAP

[redacted] - .2mls from 20ml ON into 250ml BHI

[redacted] process with half volume kirts pro[illegible]

- 5ml Tris sucr.
- .5ml lyso (bottle)
- 1ml EDTA (bottle)
- 6.5mls triton (bottle)
- spin 26K 2.5hrs. 11:30am →
- phenol
  iso chlor.

[redacted] • RNAse 10:15am
• EtOH 1:15pm

VTC harvested
[redacted] spin, wash, spin, lypho.
Assay 1ml / sample & read OD
[redacted] OD 100 ul + 1ml

	260	280	320		
HUG 1	.097	.051	.002	R 1.9	485 ug/ml
2	.107	.058	"	1.8	535 ug/ml
VHCK7	.127	.070	"	1.8	635 ug/ml
ICE	.112	.056	.008	2.0	560 ug/ml

Signed .... Date ....

2166	.20	145.000	130.000	1.115300
2167	.20	160.000	110.000	1.454500
2168	.20	125.000	95.000	1.315700
2169	.20	180.000	265.000	.679200
2170	.20	155.000	145.000	1.068900
2171	.20	115.000	85.000	1.352900

2166	1.00	137.0000	102.0000	1.343100
2167	1.00	178.0000	120.0000	1.483300
2168	1.00	141.0000	97.0000	1.453600
2169	1.00	166.0000	227.0000	.731200
2170	1.00	141.0000	121.0000	1.165200
2171	1.00	99.0000	86.0000	1.151100
2172	1.00	141.0000	112.0000	1.258900
2173	1.00	141.0000	133.0000	1.060100

glycine

100λ / 100λ

60' cyto HC6TL 10 ul/ml   1/64

TAO 1-24
TAP-1-3
TAQ-1
J550L

Run whole sample on 5% POf

1/17

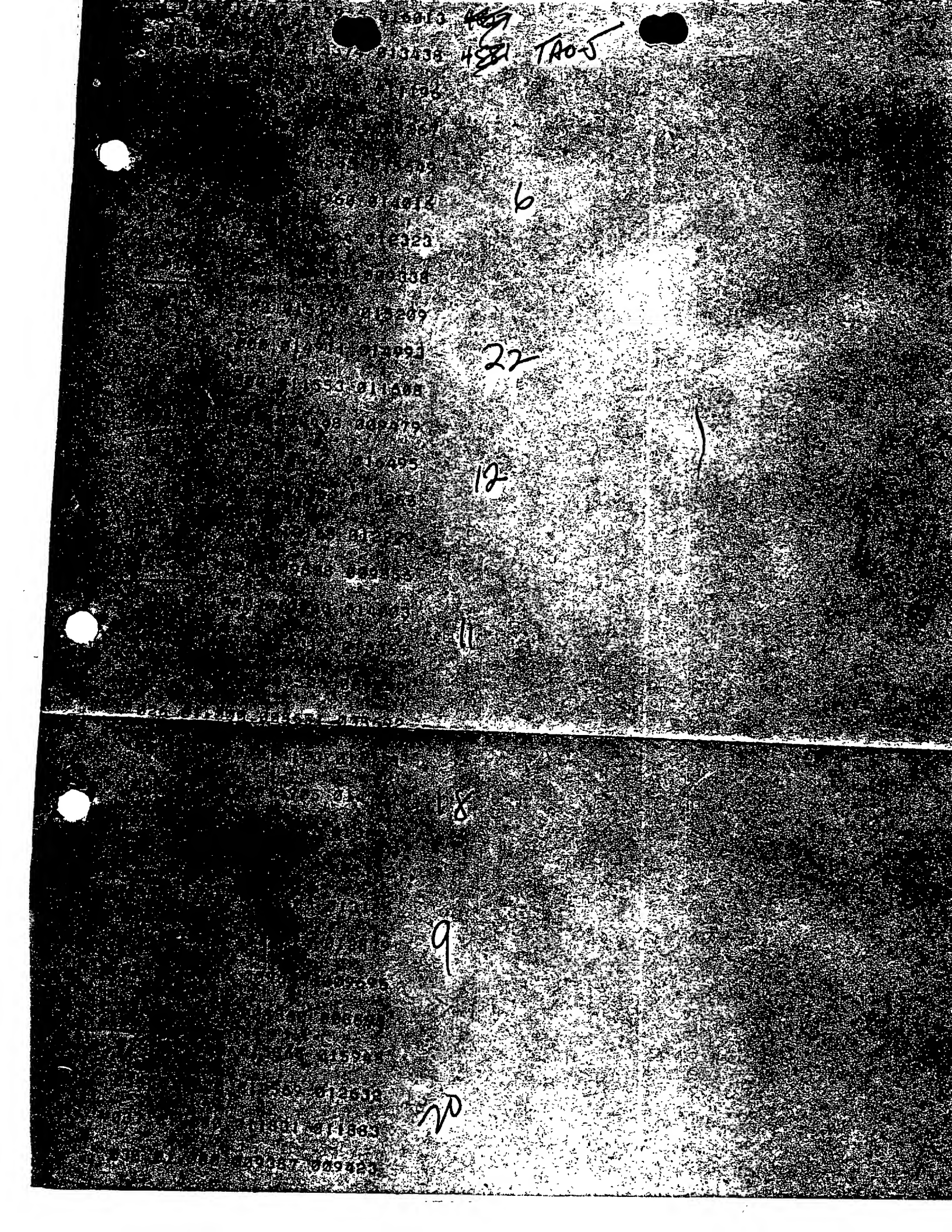
Sheep coated plates with PC-KLH + added Sups. o.n. 4°
Sups contain 20% HS
Washed plates 3X in 1% BSA in PBS
Added 50 µl of a 1/20 dil of protein A in PBS-BSA
1 hr 4°
Aspirated,
Washed 3X in PBS-BSA
Dried, cut + count
14C control went 3 hr instead of overnight

TAOJ

6

22

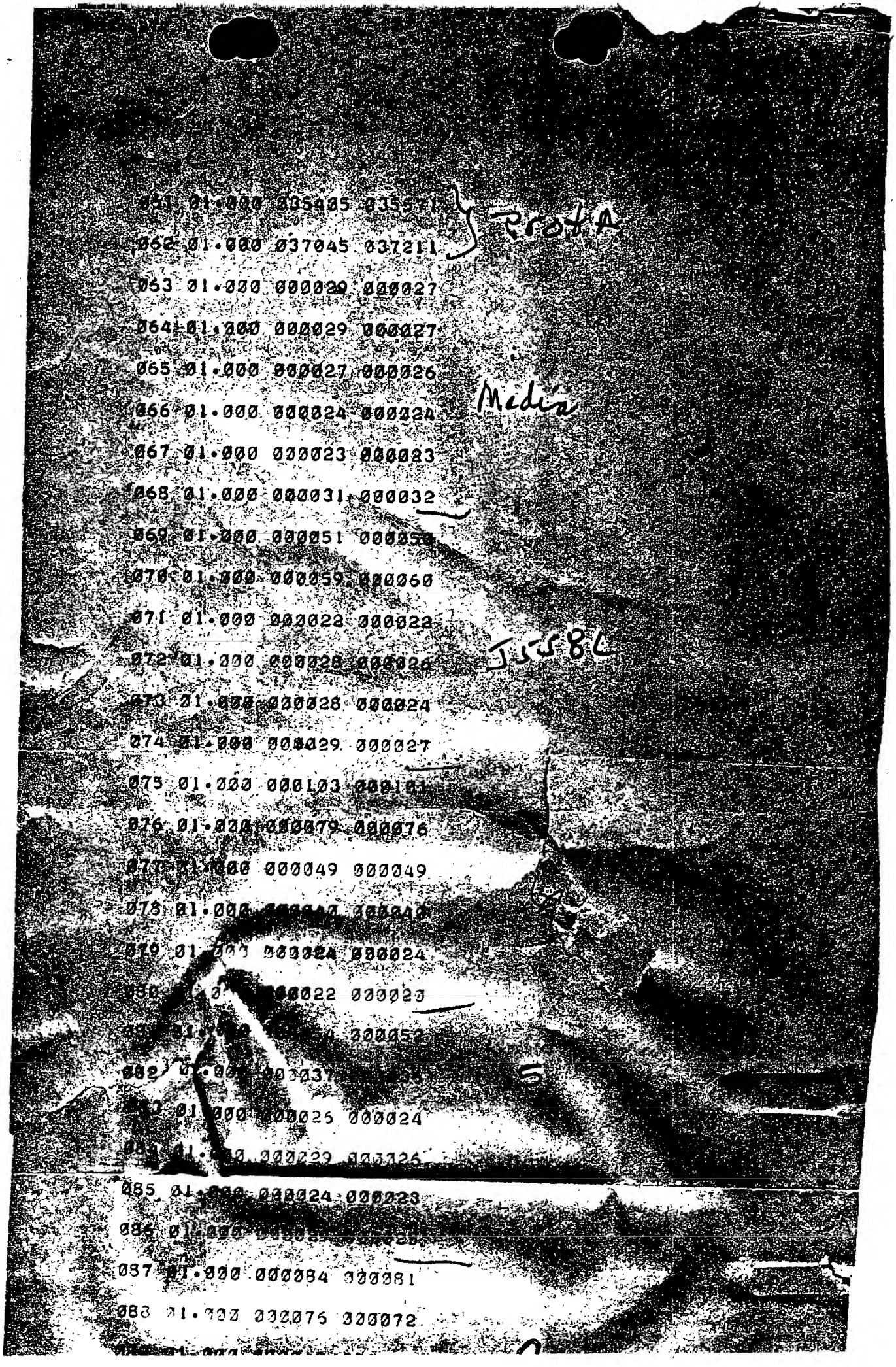
12



		st	1:2	1:4	1:8
		JSRD			
#5	1	4847	4597	6687	3583
#6	2	4483	5175	7814	4654
#22	3	4043	6152	7099.	4813
#12	4	5329	4414	7720.	5172
#11	5	2837	4524	5040	4008
#18	6	3015	5926	8587.	4048
#9	7	2000	4038	5187	4211
20	8	4782	3791	7374	4739

1/18/84

Coated well with 50λ of 1:200 PC-KLH in PBS
2 hrs rt
Washed 3X in PBS
Added 100λ of 1% BSA 30'
Added 50λ supernatant (6 hr sec - no serum) on 40
@ 1/19
Washed 3X with PBS-BSA
Added 50λ of a 1/40 dilution of protein A 40 60' in PBS-BA
S107 - added 4000 cpm ^{14}C Rat anti-Kappa
Wash 3X PBS-BSA
Wash 3X PBS
Dry
Cocount



061 01.000 035405 035571
062 01.000 037045 037211
063 01.000 000029 000027
064 01.000 000029 000027
065 01.000 000027 000026
066 01.000 000024 000024
067 01.000 000023 000023
068 01.000 000031 000032
069 01.000 000051 000050
070 01.000 000059 000060
071 01.000 000022 000022
072 01.000 000028 000026
073 01.000 000028 000024
074 01.000 000029 000027
075 01.000 000103 000101
076 01.000 000079 000076
077 01.000 000049 000049
078 01.000 000049 000049
079 01.000 000024 000024
080 01.000 000022 000020
081 01.000 000052
082 01.000 000037
083 01.000 000025 000024
084 01.000 000029 000026
085 01.000 000024 000023
086 01.000
087 01.000 000084 000081
088 01.000 000075 000072

Prota
Media

088 01.[redacted] 000076 000072

089 01.000 000049 000049

090 01.000 000039 000039

091 01.000 000036 000035

092 01.000 000026 000027

Empty 093 01.000 000020 000020

094 01.000 000043 000045

095 01.000 000048 000048

096 01.000 000037 000034

097 01.000 000032 000032

098 01.000 000025 000020

099 01.000 000045 000043

100 01.000 000052 000052

101 01.000 000031 000029

102 01.000 000034 000033

103 01.000 000027 000026

104 01.000 000021 000021

105 01.000 000036 000037

106 01.000 000049 000048

107 01.000 000034 000035

108 01.000 000025 000026

109 01.000 000035 000031

110 01.000 000025 000024

111 01.000 000059 000058

112 01.000 000059 000054

113 01.000 000033 000033

114 01.000 000034 000032

115 01.000 000024 000023

116 01.000 000033 000031

117 01.000 000069 000068

118 01.000 000045 000044

119 01.000 000034 000032

[illegible]	[illegible]	[illegible]	[illegible]0026
[illegible]	[illegible]	000035	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]58
[illegible]	[illegible]	[illegible]	[illegible]54
[illegible]	01.000	000033	[illegible]0033
114	01.000	000034	000032
115	01.000	000024	000023
116	01.000	000033	000031
117	01.000	000069	000068
118	01.000	000045	000044
119	01.000	000034	000032
120	01.000	000040	000038
121	01.000	000032	000032
[illegible]	01.000	000035	000035
123	01.0[illegible]	000030	000029
124	01.000	000033	000033
125	01.000	000029	000028
126	01.000	000223	000022
127	01.000	000032	000029
[illegible]	01.000	000024	000024

Je

22

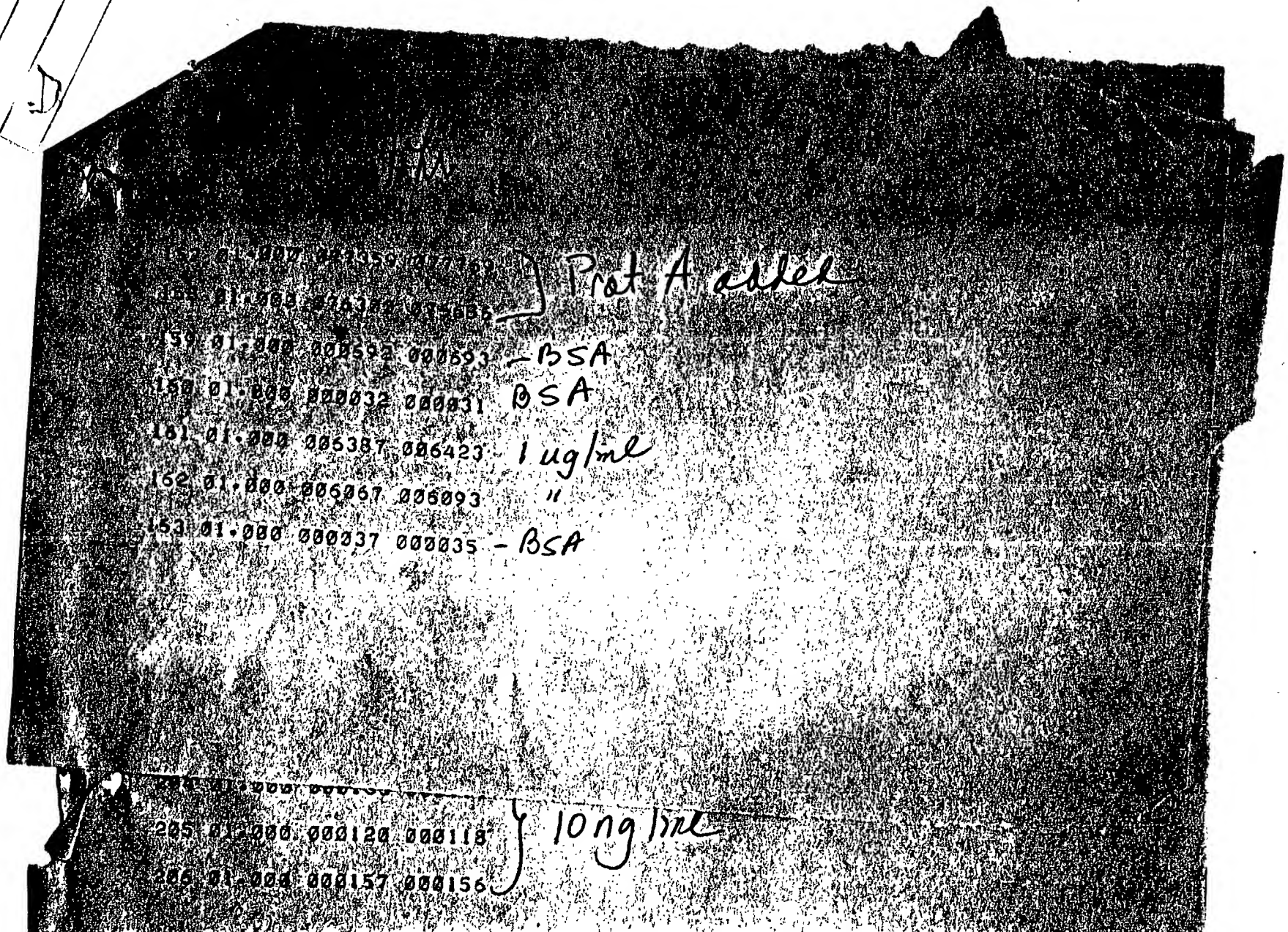
Melex + FCS

I^{125} titering 1/30

prot A received 1/27 date on it 1/30

1. Got normal human plasma from Nicki
2. Assume 10 mg/ml of IgG (7-14mg)
3. Serial dilution 1:10 down to 10 ng/ml in PBS
4. Coated wells 2 hrs with plasma r.t.
   50λ of 1 ug/ml
   100 ng/ml
   10 ng/ml
   BSA
5. Washed 3x with BSA; added 100λ BSA 2 hr r.t.
6. Added 50λ of a 1/250 dil of protein A in PBS-BSA/0.5
7. Washed 8x with 1% PBS-BSA
8. Counted 1 plate after 1 hr 4°
   " " " o.n. 4°

Results - Can detect ~~ten~~ 5 ng (3.5-7) total human IgG.
Overnight increased counts ~ 10% - ∴ 1 hr sufficient


Prot A eluate
159 01.000 000592 000593 - BSA
160 01.000 000032 000031 BSA
161 01.000 006387 006423 1 ug/ml
162 01.000 006067 006093 "
163 01.000 000037 000035 - BSA
205 01.000 000120 000118 10 ng/ml
206 01.000 000157 000156

164 01.000 006418 006448 1ug/ml
165 01.000 000885 000889 100ng/ml
166 01.000 000928 000933 100ng/ml

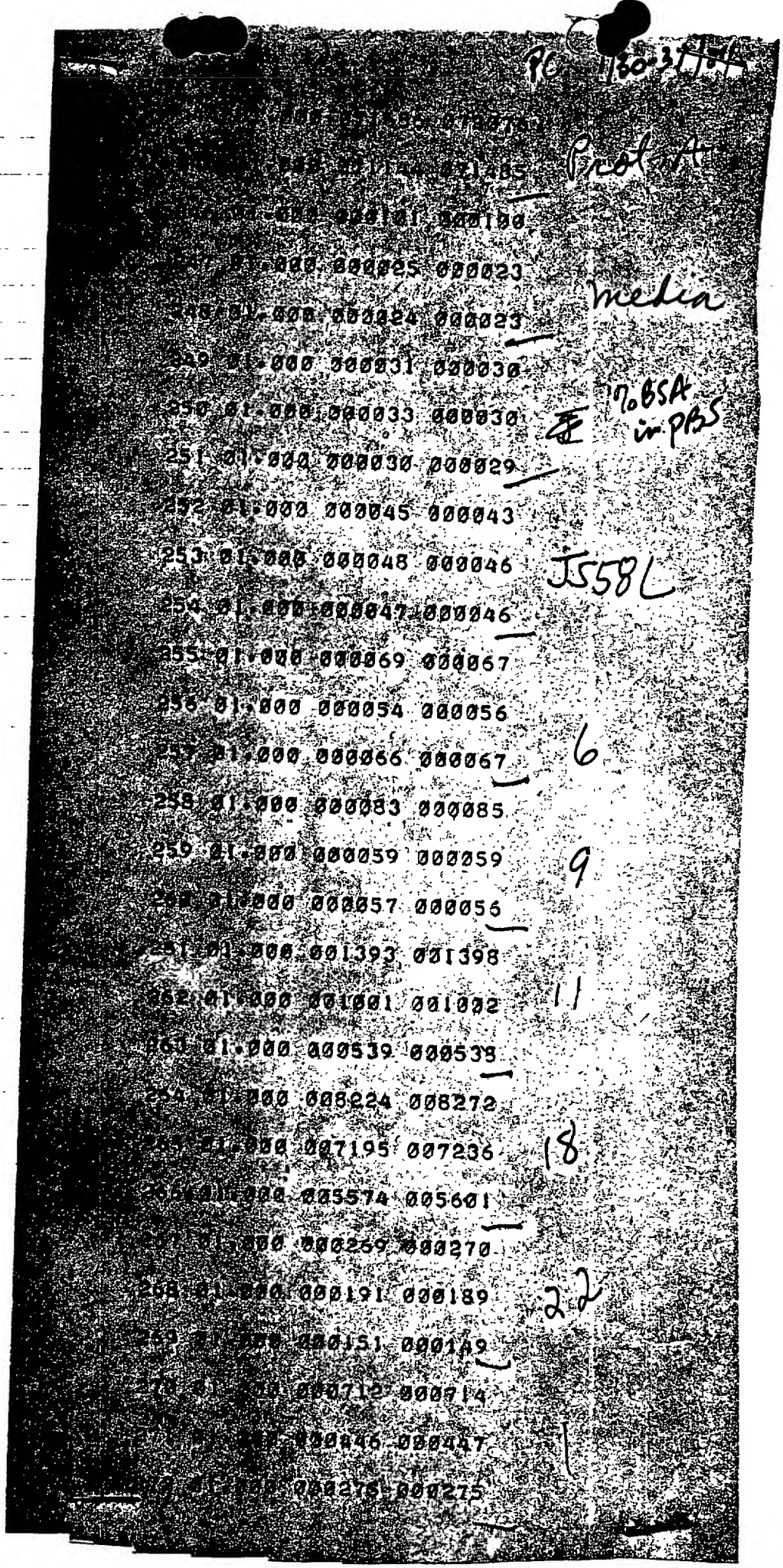
201 ... } 100ng/ml
202 01.000 001005 001011 }
203 01.000 000987 000992
204 01.000 000136 000136 } 10ng/ml
205 01.000 000120 000118 }
206 01.000 000157 000156

Ref #
BSA
1 ug/ml
100 ng/ml
10 ng/ml

I^{125} protein A PC Binding 1/20

1. Coated wells 2 hrs. r.t. with 50λ 1:200 dil PC-KLH
2. Washed 3x with 1% BSA
3. Blocked with 100λ 1% BSA 2 hrs r.t.
4. Washed 3x with 1% BSA
5. Added Supernatants 50λ serially diluted 1:2 in 1% BSA St. ; 1:2, 1:4
6. Inc. O.N. 4°
7. Washed 6x 1% BSA
8. Added 50λ of prot. A (1:250) 4° 60'
9. Wash 8x 1% BSA
10. Count

TAO-18 pos - very
TAO-11 ~5 as positive
TAO-1 slightly positive
TAO-22 - very slightly positive
All picks relatively early


Prot. #
media
1% BSA in PBS
J558L
6
9
11
18
22

2/2 /84

Overnight see label
100 ml 35 meth
~5X10⁶ TA0-18. in 3.0 mls

2/3

Harvest

2/4

20% TCA ~17,000 cpm

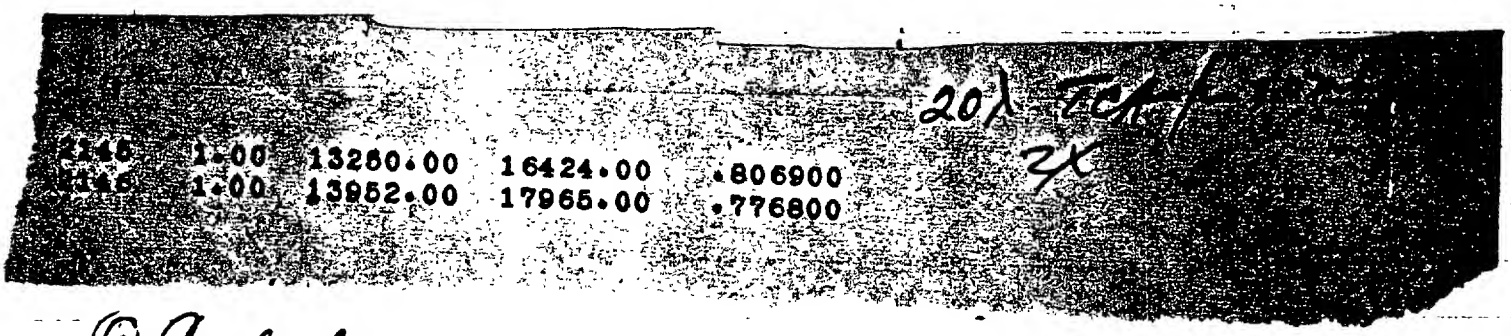
Shit!

Here are the counts on the pots. The column didn't work on the good line either. Just a mess of digits. Come off with PC and visit Eugene.

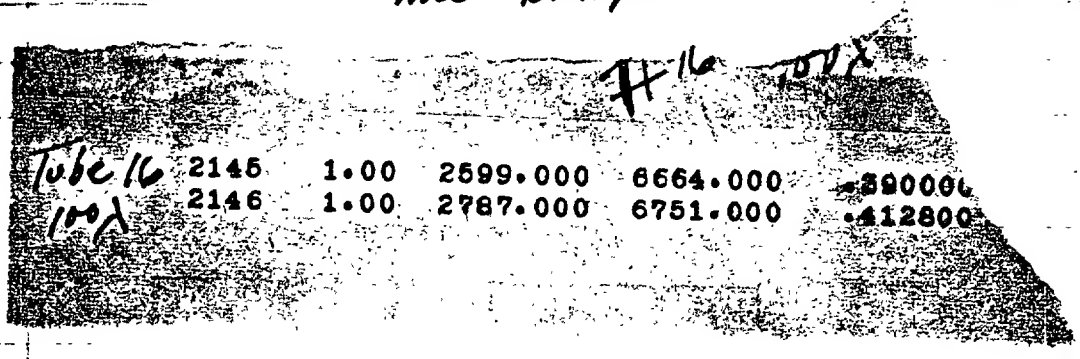
2178	1.00	155.0000	174.0000	.890800 #1	
2179	1.00	148.0000	121.0000	1.223100 #2	5λ/50λ
2180	1.00	110.0000	60.0000	1.833300 #3	
2181	1.00	8.0000	12.0000	.666600	

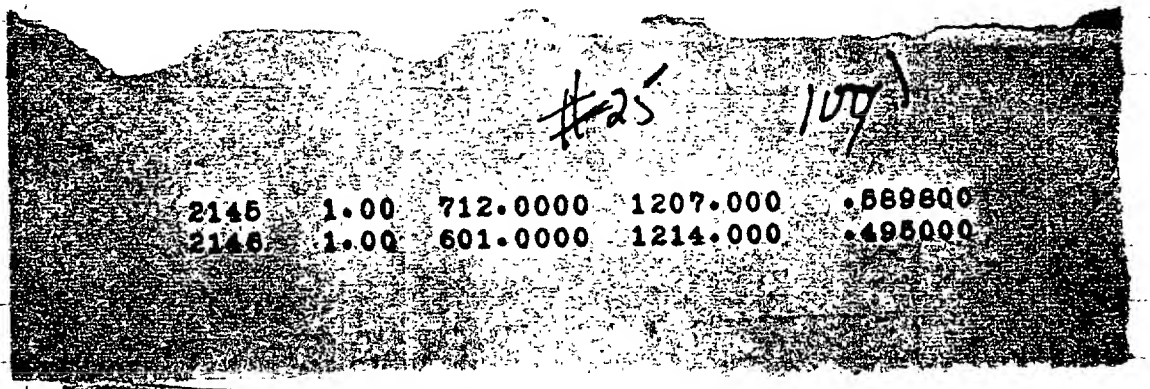
PC - Coleman 2/6/84

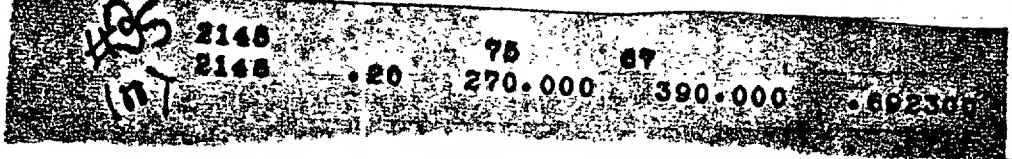
① Washed with 50 ml PBS
② Ran three 40 ml 0.1 M glycine pH 2.2
③ Brought pH back up Repacked
④ Washed with 100 ml PBS
⑤ Loaded sample in 20 ml

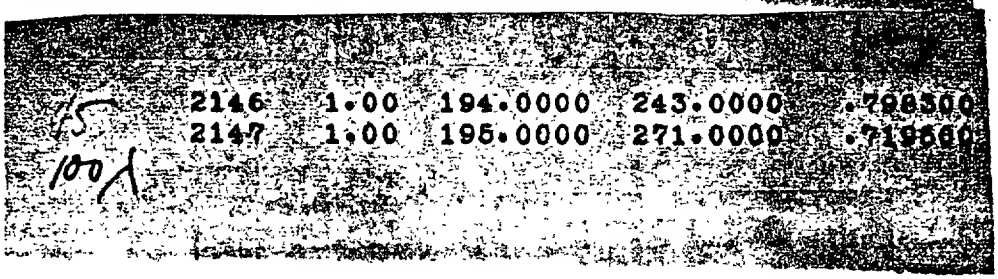


⑥ Collect ml samples









55

2147	.20	240.000	190.000	1.263100
2148	.20	170.000	120.000	1.416600

24	2111	1.00	165.0000	170.0000	.970600
25	2112	1.00	159.0000	137.0000	1.160600
26	2113	1.00	197.0000	95.0000	2.073600
30	2114	1.00	167.0000	136.0000	1.227900
30	2115	1.00	120.0000	67.0000	1.791000
34	2116	1.00	130.0000	72.0000	1.805500
36	2117	1.00	79.0000	52.0000	1.519200
38	2118	1.00	172.0000	142.0000	1.211200
40	2119	1.00	101.0000	68.0000	1.485200
42	2120	1.00	106.0000	70.0000	1.514200
44	2121	1.00	177.0000	144.0000	1.229100
46	2122	1.00	263.0000	461.0000	.570400
48	2123	1.00	158.0000	214.0000	.738300
50	2124	1.00	180.0000	259.0000	.694900

PC in Column

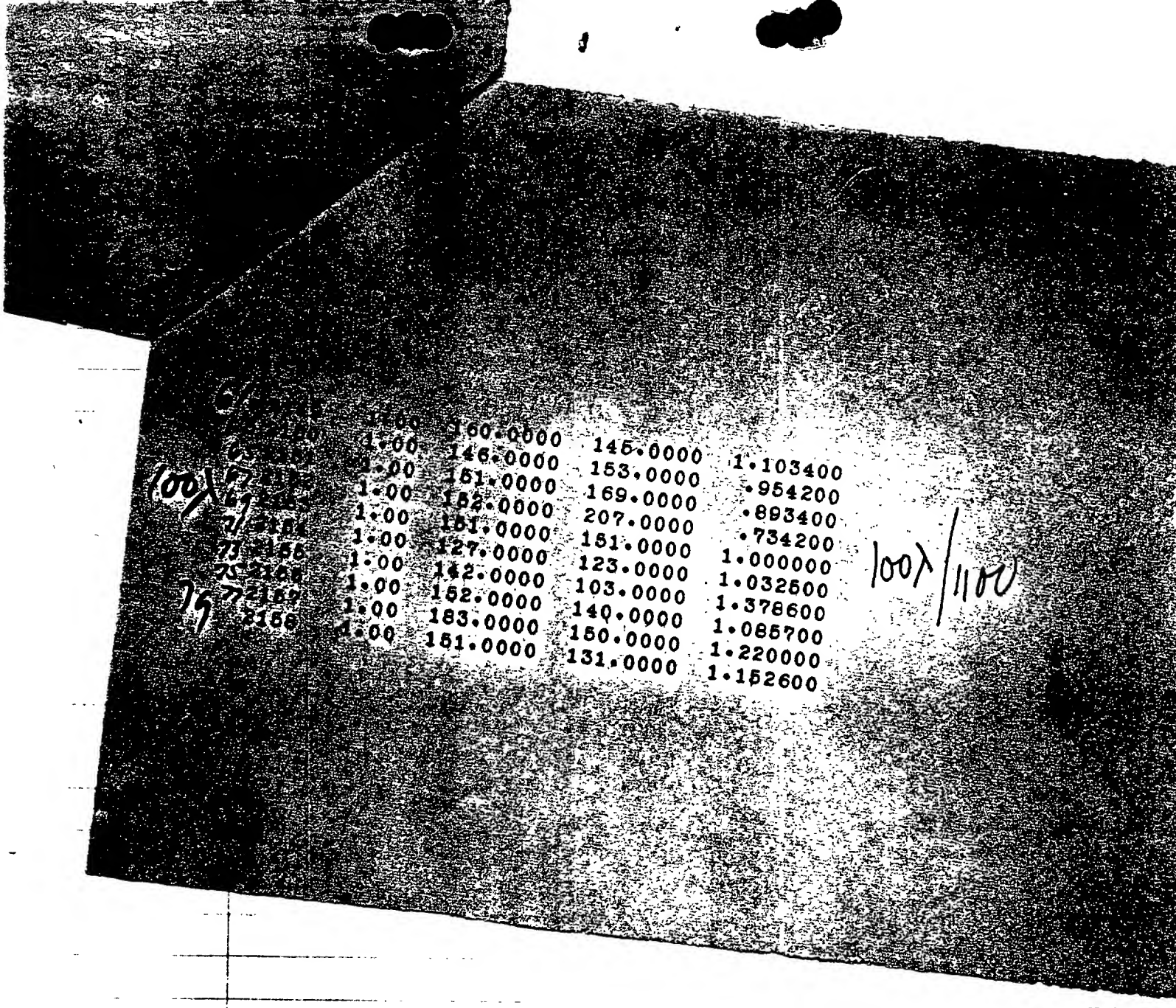
100 x /110 =

42	2129	.20	185.000	60.000	3.083300
43	2130	.20	200.000	75.000	2.666600
44	2131	.20	235.000	175.000	1.342800
45	2132	.20	165.000	245.000	.673400
46	2133	.20	205.000	215.000	.953400
47	2134	.20	155.000	160.000	.968700
48	2135	.20	155.000	220.000	.704500
49	2136	.20	175.000	190.000	.921000
50	2137	.20	230.000	190.000	1.210500
51	2138	.20	215.000	150.000	1.433300
52	2139	.20	185.000	170.000	1.088200
53	2140		38	32	
54	2141	.20	200.000	115.000	1.739100

50 x /110 =

42 2129	1.00	131.0000	76.0000	1.723600
43 2130	1.00	133.0000	104.0000	1.278800
44 2131	1.00	170.0000	163.0000	1.042900
45 2132	1.00	194.0000	248.0000	.782200
46 2133	1.00	177.0000	210.0000	.842800
47 2134	1.00	166.0000	190.0000	.873600
48 2135	1.00	160.0000	220.0000	.727200
49 2136	1.00	158.0000	212.0000	.745200
50 2137	1.00	150.0000	146.0000	1.027300
51 2138	1.00	153.0000	130.0000	1.176900
52 2139	1.00	159.0000	170.0000	.935200
53 2140	1.00	106.0000	113.0000	.938000
54 2141	1.00	142.0000	113.0000	1.256600
55 2142	1.00	179.0000	196.0000	.913200

1	.20	266.000	135.000	1.962900
2	.20	290.000	190.000	1.526300
3	.20	205.000	160.000	1.281200
4	.20	155.000	160.000	.968700
5	.20	180.000	160.000	1.125000
	52	20		
1	.20	150.000	95.000	1.578900
2	.20	110.000	155.000	.709600
3	.20	185.000	155.000	1.193600
4	.20	165.000	130.000	1.269200



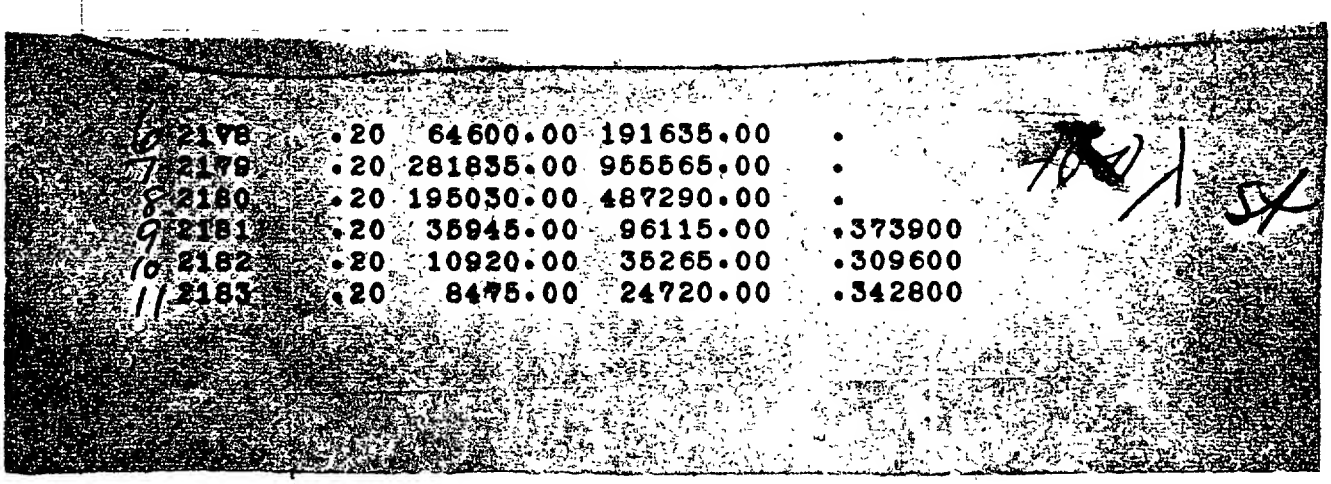
1.00	160.0000	145.0000	1.103400	
1.00	146.0000	153.0000	.954200	
1.00	151.0000	169.0000	.893400	
1.00	182.0000	207.0000	.734200	
1.00	151.0000	151.0000	1.000000	
1.00	127.0000	123.0000	1.032500	
1.00	142.0000	103.0000	1.378600	
1.00	152.0000	140.0000	1.085700	
1.00	183.0000	150.0000	1.220000	
1.00	151.0000	131.0000	1.152600	

PC column cont'd 2/7/84

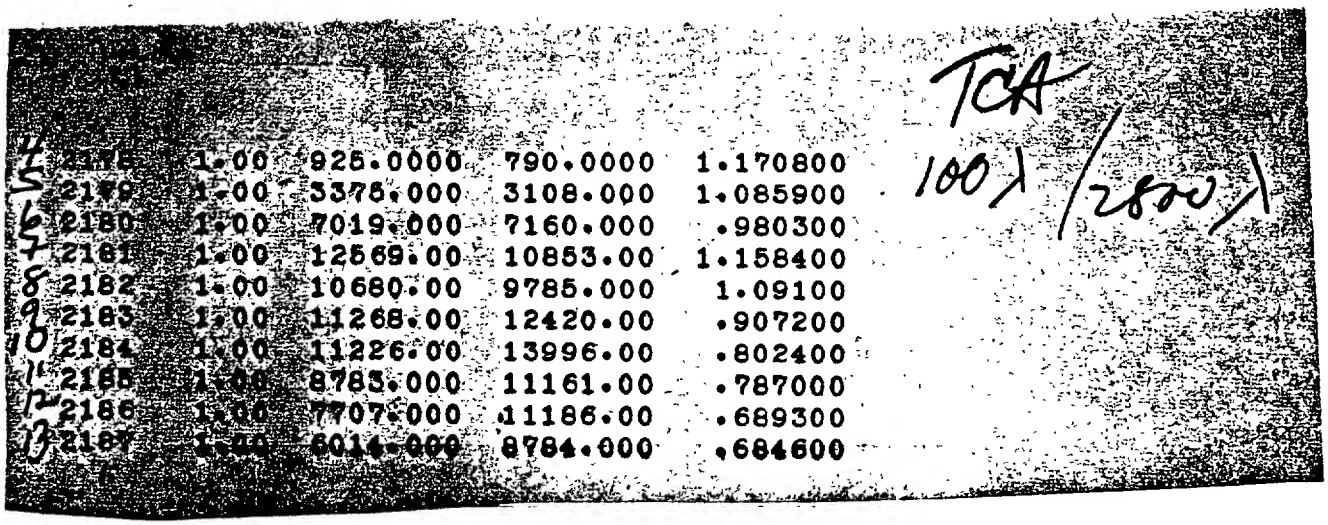
Pool - PC elevate tubes 44-49

glycine elevate 23-26

TCA what went through



6	2178	.20	64600.00	191635.00	.
7	2179	.20	281835.00	955565.00	.
8	2180	.20	195030.00	487290.00	.
9	2181	.20	35945.00	96115.00	.373900
10	2182	.20	10920.00	35265.00	.309600
11	2183	.20	8475.00	24720.00	.342800



4	2178	1.00	926.0000	790.0000	1.170800
5	2179	1.00	3378.000	3108.000	1.085900
6	2180	1.00	7019.000	7160.000	.980300
7	2181	1.00	12569.00	10853.00	1.158400
8	2182	1.00	10680.00	9785.000	1.09100
9	2183	1.00	11268.00	12420.00	.907200
10	2184	1.00	11226.00	13996.00	.802400
11	2185	1.00	8783.000	11161.00	.787000
12	2186	1.00	7707.000	11186.00	.689300
13	2187	1.00	6014.000	8784.000	.684600

PC-assay [illegible] /15

media -1 [illegible]

Heuken [illegible]

old TAO-18

NEW TAO-1
-11
-18
-22
TAO-11-7
11-8
11-9
11-11
11-12
11-13
11-14
TAO-18-1
2
4
5
7
6-1

[illegible] Prot A - use 1:190 dil
17.36 + 3282.64
PBS+BSA

275	01.000	[illegible]	[illegible]	
276	01.000	[illegible]	[illegible]	
277	01.000	[illegible]	[illegible]	
080	01.000	000042	[illegible]	
081	01.000	004033	[illegible]	
082	01.000	003759	[illegible]	
083	01.000	002695	002949	
084	01.000	003008	003061	
085	01.000	002943	002956	
086	01.000	001899	001920	
087	01.000	000034	000039	
088	01.000	000032	000029	11
089	01.000	000043	000044	18
090	01.000	000030	000027	22
091	01.000	000040	000042	11-7
092	01.000	000031	000031	8
093	01.000	000035	000034	9
094	01.000	000031	000039	11
095	01.000	000043	000042	13
096	01.000	000028	000025	14
097	01.000	000025	[illegible]	18-
098	01.000	000040	000025	
099	01.000	000032	000031	
100	01.000	000030	000029	
101	01.000	[illegible]	[illegible]	
102	01.000	000032	[illegible]	
103	01.000	000030	[illegible]	

2/21/84

200cc 35 meth from Nov
Big dish TAC-18. Transient exp with HVK 2/17/84
2% FCS

3/21

From freezer
Fresh J553 7/77
J1003-1-26 S Secretant
J1001
J1003-1-21 fresh

2/22 Sherie Put TAC-18 transient exp thru Cris PC column wash off with glycine

2/24 I counted 100 λ aliquots for ~ 40 mls

262209	1.00	230.0000	390.0000	.589700	
272210	1.00	449.0000	1142.000	.393100	Pool & dialyse against PBS
282211	1.00	416.0000	1048.000	.396900	pH ↓
292212	1.00	157.0000	183.0000	.857900	
302213	1.00	283.0000	598.0000	.473200	
312214	1.00	168.0000	296.0000	.567500	
322215	1.00	271.0000	577.0000	.469600	o.n. in cold

322209	1.00	284.0000	542.0000	.523900	pH ↑
332210	1.00	255.0000	536.0000	.475700	
342211	1.00	216.0000	446.0000	.484300	
352212	1.00	166.0000	387.0000	.428900	
362213	1.00	164.0000	403.0000	.406900	
372214	1.00	185.0000	358.0000	.516700	
382215	1.00	10.0000	4.0000	2.500000	

Pooled 27+28 - dialyze vs PBS

2/24 - Took dialyzed sample - Total vol = 8.0 ml
Added 100 µl Stock A
Rotated 4° 15'
Spun down
Washed as usual

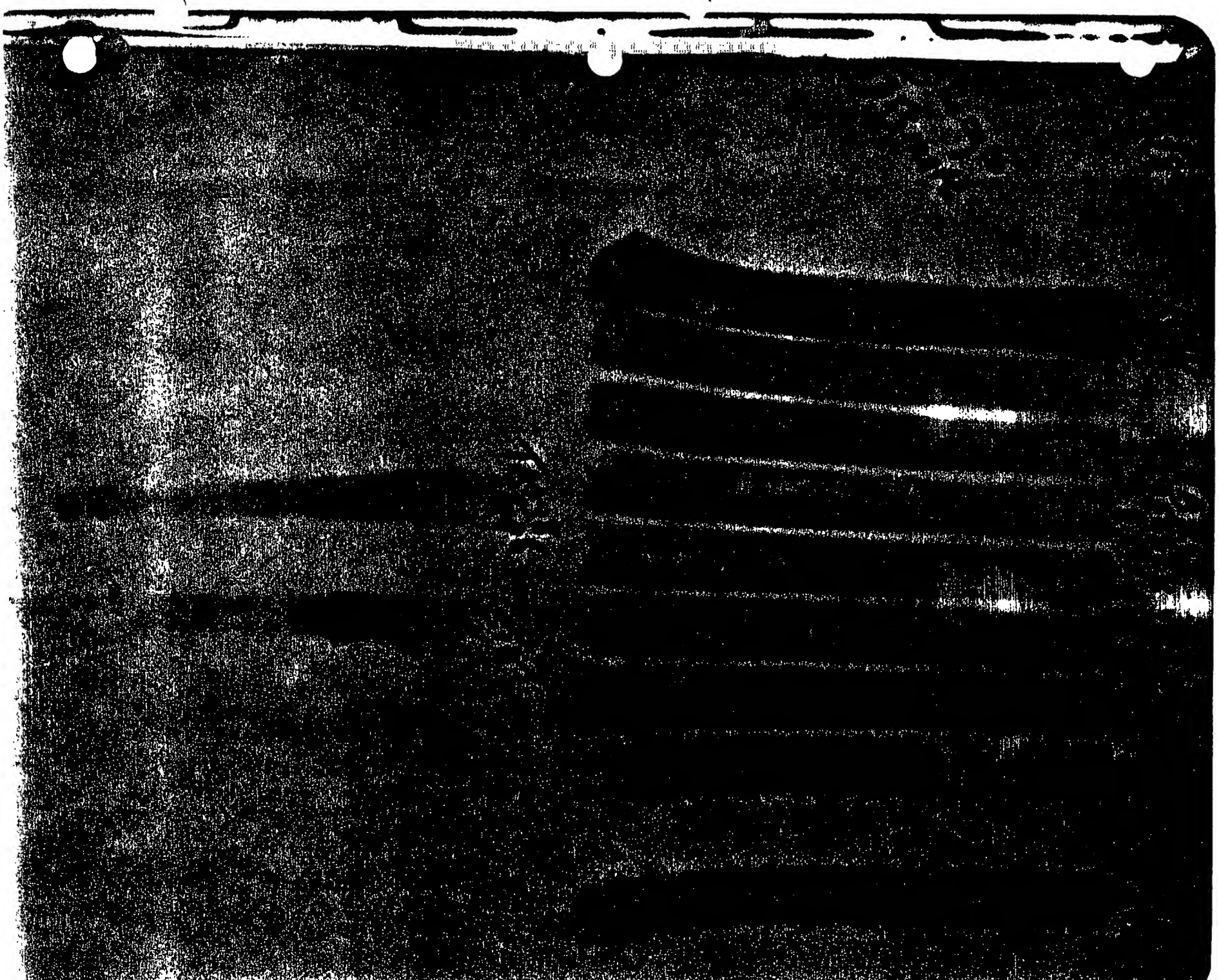
Took 300 µl of starting material
Rotated 4° 15'
Spun down washed as usual

Added 100 µl Sample buffer to each
Count 5 µl aliquots of each.

column	2232	1.00	110.0000	155.0000	.709600
	2233	1.00	120.0000	105.0000	1.142800
sample	2234	1.00	22205.00	74593.00	.297600
material	2235	1.00	28162.00	92026.00	.306000

5 µl/100 µl

Run on gel



2/8/84

Protoplasts

use 5×10^6 J558L/pt — 1×10^6 cells/ml
except HUK — ½ as many.
use 5ml protoplasts/pt
everything by std method except v. careful
to spin PEG after 1½ minutes — 8½ minute incubation at 37°

	Bacteria	OD600	Transfectants
1	VHCK	1.3	41/96
2	VHCK ΔXba Δ17	.970	62/62 (av ~5/well
3	VHCK ΔXba TAY	1.0	74/74 (av ~8/well
4	Rab 1.7	.95	9/88
5	VHCK Δ17 (TAZ)	.23	84/84 (av ~5/well)
6	232	.5	1/96
7	232A	1.2	0/96
8	232B	1.5	1/96
9	232C	1.4	2/96
10	HUK	0.6	22/43
11	HUG2	1.6	
12	pSV2	1.7	58/96 (av 1.5-2/well)

(sectors for map)

dilute all protoplasts to ideal OD of .5
were VHCKΔ17 looked v. light so added 2x volume to cells.

with HUK + HUG2 — 2½ ml of each/pt.
J558 HUG + HUK 96/96 (av ~5/well
resuspend after fusion in Iscove's 20% FCS + NYS + Gent. (100μg/ml)

use PAI 7/96
PAI.O (TBB) HUG HUK 11/96
SP2/0 996
~5×10^6 cells/pt. but didn't count.

fuse to 25 ml HUK + 2x HUG

take one SP2/0 fusion for transient

RIA

3/28/84

5ml cells open down - Washed 2X in IMDM

Resuspend in 2 ml IMDM + 10 FCS + ½ HXM

Collect sup 24 hrs later / 1:200 TC-K CH 2hr ht.; 1% BSA 2hr RT

RIA - 10% HS

PAI

TBB-1

2

3

5

TBC-1

2

3

5

PAI

PAI

10 HS

BB-1

BB-2

BB-3

BB-5

BC-1

BC-2

BC-3

BC-5

2/21/84

Ligations:

(1) 20 µl BK - intermediate
- 2 µl NaCl
50 µl EtOH
pick - mini

(3) - 5 µl HUK linear
- 5 µl Bgl 2 100
- 1 µl NaCl
20 µl EtOH
pick - grunstein

(4) ! 40 µl Δ72 R1+K 72
! 20 µl 232 R1+K
1 µl RNA
6 µl NaCl 7
pick - mini

(5) ! 20 µl Lab 1.7
5 µl Δ72 2.8
1 µl RNA
4 µl NaCl 29
pick - mini

(6) 720 µl 6-kb Rab
- 5 µl 232 Bam
1 µl RNA
3 µl NaCl 850
pick for Grunstein

- 2 µl pSV2 neo 140
(2) - 10 µl HUK insert
- 2 µl ~~EtOH~~ NaCl
50 µl EtOH
pick mini

spin down
decant
dry in dessicator

add 88 µl H2O
2 µl 10X ligase
.3 µl ligase

begin ligation at 2:50

note: clearly lost in ppt. ligation

Controls

(7) 1 µl pSV2 neo 28
(8) 5 µl HUK insert 200
(9) 2 µl HuK linear 400
(10) 2 µl 232 Bam 207

3/11

	1	2	3	4	5	6	7	8	9
1	30 µl R7				5 µl pSV2 Neo			10 µl H U K	
2									
3	70 µl H$_2$O				85 µl H$_2$O			80 µl H$_2$O	
4	10 µl 10x BglII				10 µl 10x Bam			10 µl 10 x Bam	
5	3 µl BglII				2 µl Bam			2 µl Bam	
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									
21									
22									
23									
24									
25									
26									
27									
28									
29									
30									
31									

UK - R1 complete
Bam partial
second because
don't know which
pairs are useful

Prep for cloning

① 4 µl pSV2 neo
5 µl IBI - buffer C
41 µl H_2O
1 µl R1 (16 u
1 µl Bam (20 u)
digest 1 hr - 37°

② 10 µl HVK
5 µl IBI - buffer C
40 µl H_2O
1 µl R1 - digest 1 hr
1 µl Bam - digest 15'
add ficoll & VP.

③ 5 µl Δ72
5 µl 10 x IBI buffer C
40 µl H_2O
1 µl R1
digest 1 hr. 37°

④ 10 µl P7
5 µl IBI - Bgl buffer
35 µl H_2O
1 µl Bgl II
digest 4 hrs - 37°
p7 did not cut with Bgl
discard

⑤ 5 µl Δ72Bld
5 µl 10 x Bam
40 µl H_2O — 1 µl Bam
digest 15 minutes

⑥ 10 µl HVK
5 µl IBI - buffer C
35 µl H_2O
1 µl Bam
digest 37° - 1 hr.

⑦ 4 µl pSV2 neo
5 µl IBI - buffer C
41 µl H_2O
1 µl Bam
digest 37° 1 hr.

1st ligation

isolate half of HUK insert

1/6 of pSV2 neo – BAm

isolate into 100 µl

ppt ① 10 µl pSV2 neo + 30 µl HUK — 10/600 70/200

② 10 µl pSV2 neo

③ 30 µl HUK

resuspend in 30 µl ligation buffer

add 0.1 µl ligase to ① 1

3/21 use 15 µl to transform HB101 Madison 3/23 by Ken

also recover remainder of pSV2 neo and HUK insert

Gto H ppt. 10 µl Neo

10 µl Neo

10 µl Neo + 100 µl HUK

} total vol of pSV2 neo and HUK about 300 µl each

resuspend in ligation buffer

1/300, 100/300

1/30 + 1/3

no colonies for pSV2 neo no ligase

1/10 : 3/20 >100 HUK + neo

~200 Neo + ligase (3/24)

3/28

Protoplasts

NeoHuK3	0.8
Hug 2	.44
NeoHuK1	0.85

mustake - used ½ normal amount of lyozyme
otherwise used std procedure
3 x 10⁶ cells / well - 25 ml protoplasts
plate 2 wells into 2 plates per
3/29 add each expt combination

cells TAO-6
TAO-18
PAIO

do TAO with HUA a HUK3
do PAIO with HUK1 a 3 + HUG2

3/30 put selective medium into all microdishes

to half of big dishes resuspend in labeling medium + ½ µl ^{35}S-meth + 2% FCS
3/31 spin cells out
also make RNA from TAO

PAIO looked dead so discarded them

forgot and left in autoclave

5/2 Plates

		PAIO - HUG2 + HUK1 / HUG2 + HUK3	no colonies / no colonies
TAO-18	+ HUK1	1	1
TAO-18	+ HUK3	0	9
TAO-6	+ HUK1	29	26 (#27 from this plate)
	+ HUK3	31	27

long term secretion of HUGT Neo-Huk clones

Clones incubated O.N. in 5μC ^{14}C-UTL - 2 ml
~ 10^6 cells/incubation

Secretion run through Eric's PC column -
note: previously had used PBS as buffer but
Eric says PBS can interfere with PC
binding - switched to Tris-saline buffer.

2254	2.00	196.5000	408.5000	.481000	tube #10 → gradient
2255	2.00	171.0000	387.0000	.441800	
2256	2.00	125.5000	253.0000	.496000	
2257	2.00	116.5000	269.5000	.439700	
2258	2.00	113.5000	205.5000	.552300	
2259	2.00	117.5000	207.5000	.566200	
2260	2.00	128.5000	272.5000	.471500	
2261	2.00	345.0000	175.5000	1.965800	
2262	2.00	121.5000	254.5000	.477400	
2263	2.00	91.0000	129.5000	.702700	
2264	2.00	116.5000	121.5000	.958600	
2265	2.00	81.5000	91.0000	.895600	
2266	2.00	96.5000	129.5000	.745100	

~1 ml of #27 eluate 15 ml 10^{-2}M-P

Pools

2-8

9-12

13-20

Pools made of fractions 2-8, 9-12, 13-20
add 70μl staph A to each fraction
spin down
wash 3X in tris-saline
add 100μl sample buffer - boil
centrifuge
count 5μl

2263	1.00	96.0000	107.0000	.728900	13-20
2264	1.00	135.0000	202.0000	.666300	9-12
2265	1.00	288.0000	892.0000	.323300	2-8

Also to test binding raw secretion from remaining clear through column. Secretion were pooled.

worked extensively with this column eluted with 20 ml of 10^{-2} M PC.

1 2068		287	184	
2 2069	1.00	169.0000	183.0000	1.032200
3 2070	1.00	98.0000	151.0000	.649000
4 2071	1.00	111.0000	154.0000	.720700
5 2072	1.00	88.0000	131.0000	.671700
6 2073	1.00	136.0000	279.0000	.487400
7 2074	1.00	255.0000	669.0000	.370100
8 2075	1.00	304.0000	1089.000	.279100
9 2076	1.00	304.0000	1011.000	.300600
10 2077	1.00	181.0000	537.0000	.337000
11 2078	1.00	135.0000	274.0000	.492700
12 2079	1.00	109.0000	189.0000	.576700
2080	1.00	61.0000	119.0000	.512600
2081	1.00	82.0000	120.0000	.683300
[illegible]	1.00	4.0000	3.0000	1.333300

PC applied to column

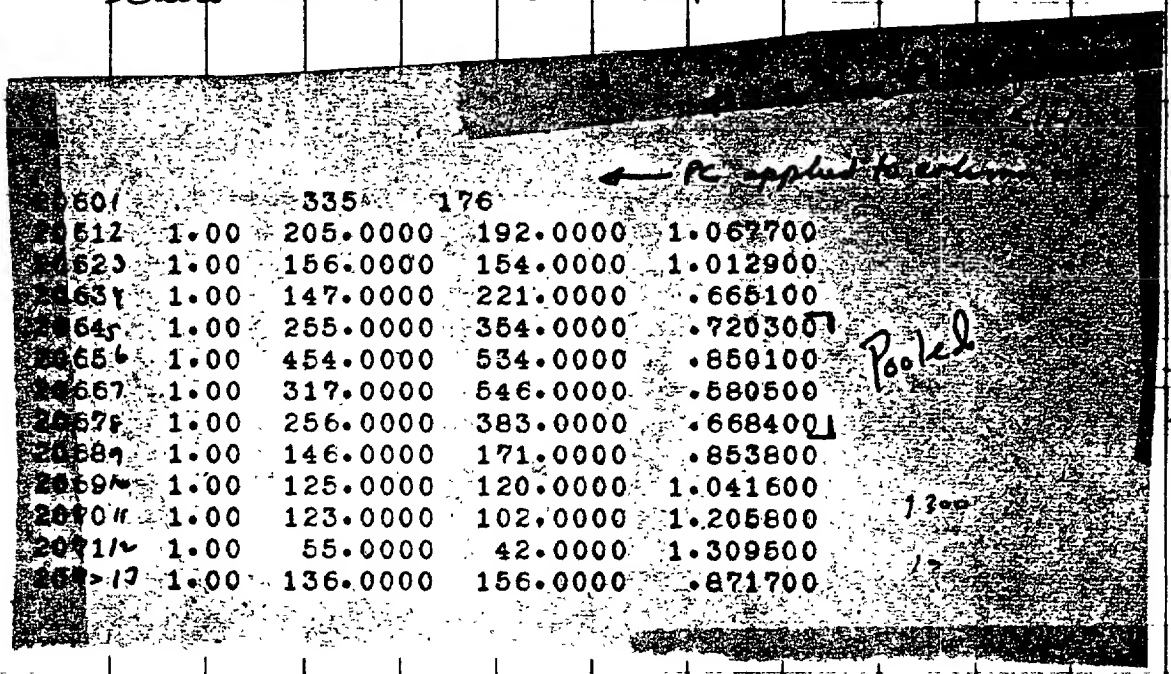
Pooled 6-11

add 200µl anti-K Sepharose

5/14

Attempt to purify chimeric PC binding proteins

overnight label of TBO-6 + Neottak-27
20 μl of ^{14}C-UTL
about 4×10^6 cells - in 5 ml
spin out cells
apply about 3 ml of PC-Sepharose
wash with Tris-saline
elute with 10^{-2}M PC - 20 ml



← PC applied to column

20601		335	176	
20612	1.00	205.0000	192.0000	1.062700
20623	1.00	156.0000	154.0000	1.012900
20634	1.00	147.0000	221.0000	.665100
20645	1.00	255.0000	354.0000	.720300
20656	1.00	454.0000	534.0000	.850100
20667	1.00	317.0000	546.0000	.580500
20678	1.00	256.0000	383.0000	.668400
20689	1.00	146.0000	171.0000	.853800
20690	1.00	125.0000	120.0000	1.041600
20701	1.00	123.0000	102.0000	1.205800
20712	1.00	55.0000	42.0000	1.309500
20723	1.00	136.0000	156.0000	.871700

Pooled

1300

~5 ml fractions

material eluted by PC - pooled as indicated

200 μl of Staph A added to flow through material centrifuged in Sorvall

10 ma

48 - Tao-6 Hhuk - S107

5/14

~~Houghton~~

assume 1 µg/1 ml
Staph burst 2 mg/ml

T139
T102
T156

Wash Staph 3x in PBS

Take 5 ml of culture Super; add .5 ml 1M Tris pH 8
incubate with .5 ml Staph
also 200 µl of ~~anti-human K~~ R4 - 1→2 - work 4x
spin out Staph - ~~work x2 in PBS Tris Saline~~
pH 4.8
5 µl Monoclonal
add 200 µl NMS
incubate 20 minutes
spin - out
work 3 x in Tris saline,
resuspend to 0.5 ml

200
10
2 mg

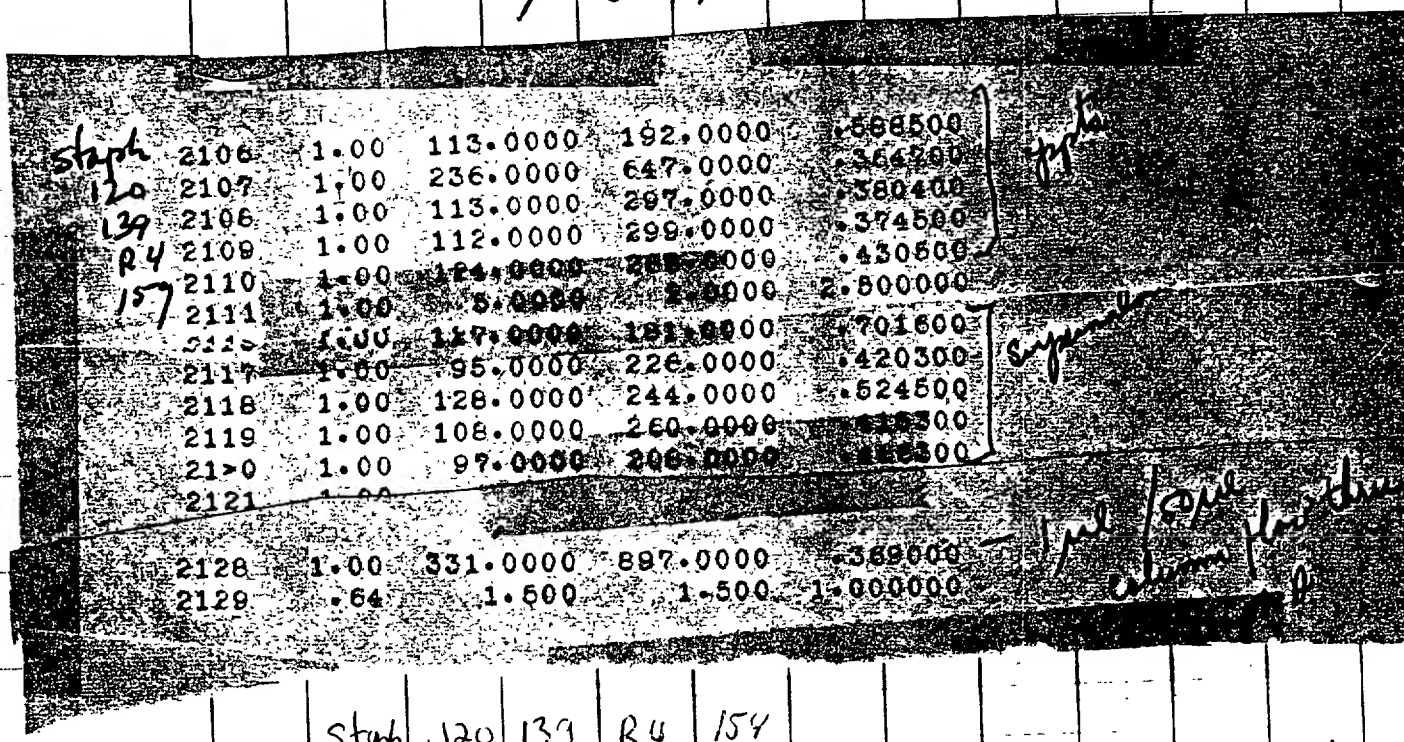
Take culture supernants S107
TA06
45.6

① 0.5 ml + Staph A
② + Staph A + anti-Id
③ +
④ +
⑤ + Pc-Sepharose
+ anti-Kappa

40

5/7

Take 14 ml of material bound to PC column and eluted with PC
add 50 µl of Staph either uncoated or coated as prepared on 5/4
incubate together
wash 4x in Tris-saline
dissolve in 60 µl of SDS sample buffer - count 5 µl
also count 200 µl of supernatant



staph	2106	1.00	113.0000	192.0000	.588500	ppt
120	2107	1.00	236.0000	647.0000	.364700	ppt
139	2108	1.00	113.0000	297.0000	.380400	ppt
R4	2109	1.00	112.0000	299.0000	.374500	ppt
154	2110	1.00	124.0000	288.0000	.430500	ppt
	2111	1.00	5.0000	2.0000	2.500000	
	2115	1.00	127.0000	181.0000	.701600	supernatant
	2117	1.00	95.0000	226.0000	.420300	supernatant
	2118	1.00	128.0000	244.0000	.524500	supernatant
	2119	1.00	108.0000	260.0000	.415300	supernatant
	2120	1.00	97.0000	206.0000	.470800	supernatant
	2121	1.00				
	2128	1.00	331.0000	897.0000	.369000	1 µl/spot on column flow through
	2129	.64	1.500	1.500	1.000000	

	Staph	120	139	R4	154
total ppt	2400	~~1000~~ 7800	3600	3600	3600
total remaining	360	1582	1700	1820	1400
cts ppt from flow through					45,000

11/24/83

Transient expression Experiment
Stable Transformants with Chimeric gene

Bacteria grown by standard method

		use 7 ml, discard 2	
S107HUK	.740		
VHCK ΔX6	1.0	10	5
VHCKΔ17ΔX6a	.946	9.5	4.5
VHCK 7	.560	5	
VHCK Δ17	.680	6.8	1.8
S107 16	1.5	15	10
V4-HUG2	.92	9	4

Protoplasts by std method

Cells		Resuspend DME
Y5606	20 x 30	6 ml
45.6	50 x 15	7.5
X63	40 x 10	4
JJ58	80 x 10	8
GD36	40 x 30	
JJ58	40 x 45	
W3129	15 x 10	1.6

For fusion add 1.5 ml cells* + 1.5 ml protoplasts / well
Spin down
Aspirate
Add 1 ml PEG
Spin 1.6 - 1.5 minutes
Dilute 7 ml DME
Spin 5 min
Aspirate
Resuspend 5 ml IMDM + 20% HS (except GD36 + FCS)
+ Gentamycin
Pipette vigorously to resuspend

*Except X63 use 1 ml cells - 1 ml protoplasts
W3129 0.4 0.4

PROPOSAL TO BECTON-DICKINSON

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TRANSFECTION OF CHIMERIC IMMUNOGLOBULIN GENES INTO LYMPHOID CELLS